A. M. A.
ARCHIVES OF
PATHOLOGY

AUGUST 1953
VOLUME 56 NUMBER 2

Published Monthly by
AMERICAN MEDICAL ASSOCIATION
535 NORTH DEARBORN STREET • CHICAGO 10, ILLINOIS

Entered as Second Class Matter Jan. 20, 1926, at the Postoffice at Chicago, Under the Act of March 3, 1879. Annual Subscription, $8.00

TABLE OF CONTENTS FIRST PAGE

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COMPARATIVE STUDY OF THE REACTION TO INJURY

II. Hypervitaminosis D in the Frog with Special Reference to the Lime Sacs

HANS G. SCHLUMBERGER, M.D.
AND
DONALD H. BURK, A.B.
COLUMBUS, OHIO

THE INJURIOUS effect of excessive doses of vitamin D has been studied chiefly in man,[1] rats,[2] guinea pigs,[3] dogs,[4] and chickens.[5] In these animals, as in nearly all other vertebrates, the normal calcium depot is found in the bones. Hopkins and co-workers[6] state that the extracellular fluid is "constantly being equilibrated with calcium which is resident in the vast surfaces of the skeletal lime salts." However, all parts of the skeleton do not serve equally as a storehouse of readily available calcium. Years ago, Bauer and co-workers[7] showed that the bone trabeculae, rather than the cortex, play this role; recently, Singer and Armstrong confirmed these findings by the use of radiocalcium.[8] In the female of some birds

From the Department of Pathology, Ohio State University College of Medicine.

Read in part at the meeting of the American Society for Experimental Pathology, in New York, April 16, 1952.

This investigation was supported in part by a grant from the National Cancer Institute, United States Public Health Service.

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2. (*a*) Gough, J.; Duguid, J. B., and Davies, D. R.: The Renal Lesions in Hypervitaminosis D: Observations on the Urinary Calcium and Phosphorus Excretion, Brit. J. Exper. Path. **14**:137-145, 1933. (*b*) Ham, A., and Portuondo, B. C.: Relation of Serum Calcium to Pathologic Calcifications of Hypervitaminosis D, Arch. Path. **16**:1-14, 1933.

3. Grauer, R. C.: Production of Osteitis Fibrosa with Overdoses of Vitamin D, Proc. Soc. Exper. Biol. & Med. **29**:466-467, 1932.

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5. Correll, J. T., and Wise, E. C.: The Comparative Toxicity of Calciferol, A.T. 10, and Cod Liver Oil Concentrate for Chicks, J. Nutrition **26**:641-648, 1943.

6. Hopkins, T.; Howard, J. E., and Eisenberg, H.: Ultrafiltration Studies on Calcium and Phosphorus in Human Serum, Bull. Johns Hopkins Hosp. **91**:1-21, 1952.

7. Bauer, W.; Aub, J. C., and Albright, F.: Studies of Calcium and Phosphorus Metabolism: A Study of the Bone Trabeculae as a Readily Available Reserve Supply of Calcium, J. Exper. Med. **49**:145-161, 1929.

8. Singer, L., and Armstrong, W. D.: Retention and Turnover of Radiocalcium by the Skeleton of Large Rats, in Metabolic Interrelations: Transactions of the Third Conference, New York, N. Y., January 8-9, 1951, edited by E. C. Reifenstein Jr., New York, Josiah Macy Jr., Foundation, 1951.

there is deposition of medullary bone during the preovulatory period, followed by its complete disappearance after the intensive use of calcium in the egg-laying period.[9]

In the frog the problem of the calcium depot is further complicated by the presence of a variable store of calcium in the paravertebral lime sacs.[10] These structures represent an enormous expansion of the endolymphatic sac. During larval development the sac grows into the cranial cavity and encircles the hind brain; from there, bilateral extensions pass down the entire length of the spinal canal between two lamellae of the dura.[11] At the level of the intervertebral foramina lateral diverticuli of the sac pass through the foramina with the spinal nerve roots and envelop the spinal ganglia, forming the so-called "lime sacs" (Fig. 1).

These are not the simple "sacs" that their name would imply; they are multiloculated gland-like structures lined by cuboidal epithelium (Fig. 2). When the sacs

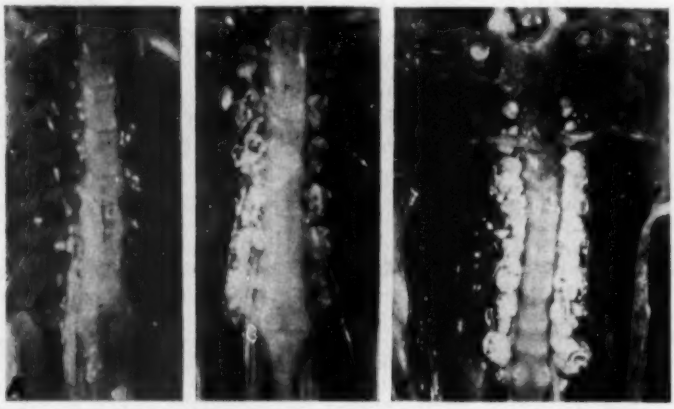

Fig. 1.—*A*, ventral aspect of the vertebral column of species of Rana pipiens showing usual appearance of the lime sacs. These are the flat, disc-shaped structures lying adjacent to the bodies of the vertebrae and between their transverse processes. *B*, exceptionally prominent lime sacs occurring under natural conditions. *C*, greatly enlarged lime sacs of a frog kept in 0.8% $CaCl_2$ during the previous 38 days and receiving 50,000 units of vitamin D on the 17th, 24th, and 31st days. Part of the skull has been removed, exposing the U-shaped extension of the calcium containing endolymphatic sac at the base of the brain. The two spherical otoliths are also shown.

are distended, the epithelium is greatly flattened; at no time were calcium deposits seen in the cells. The calcium is probably secreted by the epithelial cells into the fluid of the endolymphatic sac where it precipitates, usually in the form of micro-

9. Ringoen, A. R.: Deposition of Medullary Bone in the Female English Sparrow, Passer Domesticus (Linnaeus), and the Bobwhite Quail, Colinus Virginianus, J. Morphol. **77**:265-283, 1945.

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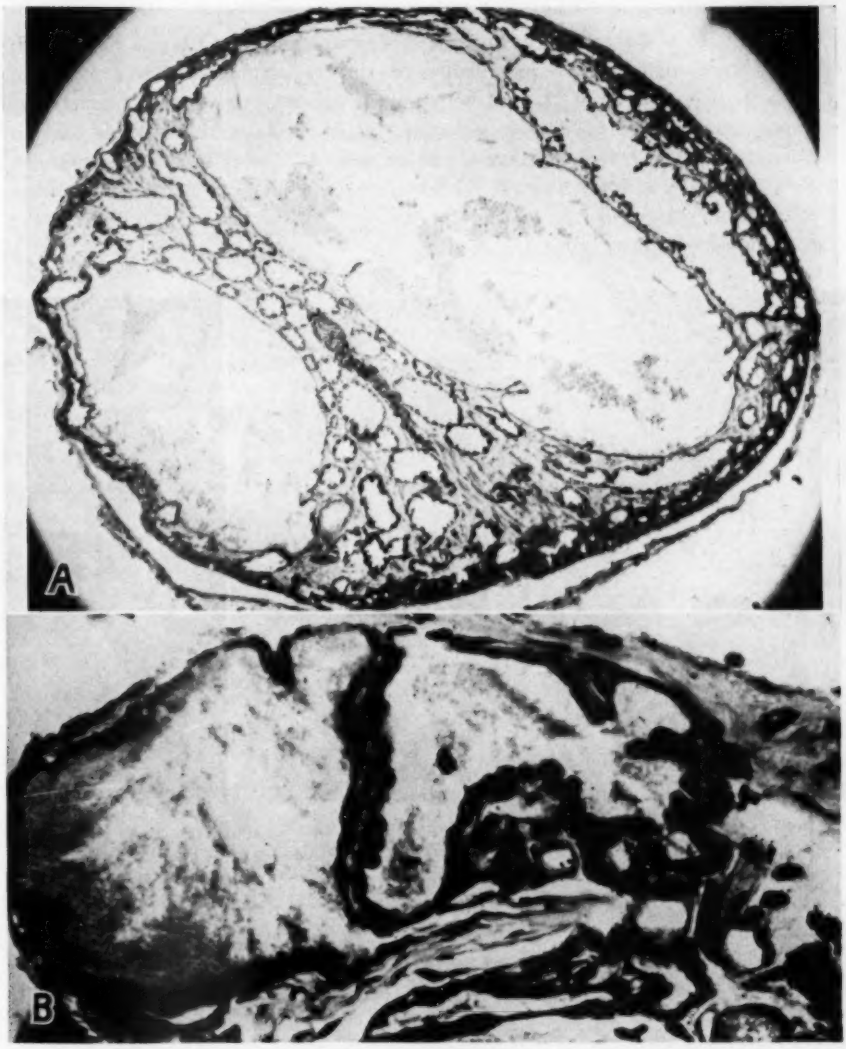

Fig. 2.—*A*, section through a lime sac removed six days after the frog had received 50,000 units of vitamin D. Though most of the calcium washed away during preparation, some granular $CaCO_3$ is still present. Part of a spinal nerve trunk lies in the parenchyma between the two largest spaces. Hematoxylin and eosin; × 100. *B*, part of lime sac from a normal frog. The somewhat flattened cuboidal epithelium has a deeply staining polychromatic cytoplasm and chromatin-rich nuclei. Hematoxylin and eosin; × 300.

scopic crystals [12] (Fig. 3). That the cells of the lime sacs have this function is further evidence of their origin from the epithelium of the labyrinth which in all vertebrates forms the otoliths (statoliths).

About 93% of the calcium in the lime sacs is present as $CaCO_3$ in a form which x-ray diffraction studies have identified as the mineral aragonite.[13] The presence of such large deposits of $CaCO_3$ in a vertebrate is unique. Among the invertebrates calcium occurs almost solely as a carbonate, but in the vertebrate animals calcium deposits are nearly always phosphates.[14] In the frog, as in other vertebrates, approximately 85 to 90% of the calcium in the bones or in pathological calcifications of the soft tissues is present as calcium phosphate.[15] Only in the lime sacs and otoliths is the calcium found as a carbonate.

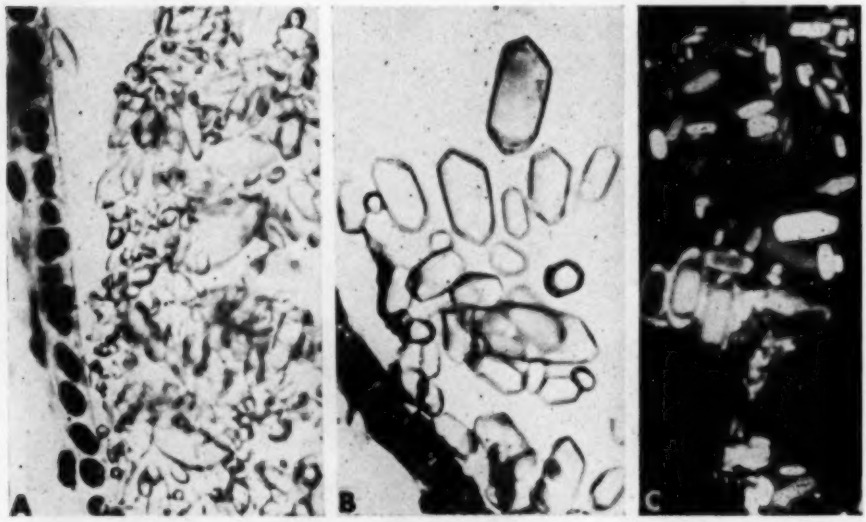

Fig. 3.—*A*, section of endolymphatic duct showing cuboidal epithelial lining and lumen filled with aragonite; × 400. *B*, orthorhombic crystals of aragonite in the endolymphatic duct; × 500. *C*, crystals of aragonite taken from a lime sac and photographed in polarized light; × 450.

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MATERIALS AND METHODS

The animals used were the leopard frog, Rana pipiens, and the African clawed toad, Xenopus laevis. The experiments were always undertaken shortly after receipt of the animals. These were then in a good state of nutrition, and subsequent feedings could be omitted, aiding greatly in standardizing conditions of the experiment.

Vitamin D was injected into the muscles of the right thigh or into the coelomic cavity. The product employed was Dee-Ron (Warren-Teed Products Company), of which each milliliter contains 500,000 U. S. P. units of vitamin D_3 in sesame oil. Crystalline vitamin D_3 (activated 7-dehydrocholesterol) [16] was dissolved in sesame oil so that each milliliter contained 500,000 U. S. P. units of the vitamin. The method of administration was similar to that employed with vitamin D_3. The tap water in which the frogs were kept contained 18 to 23 parts of calcium per million. When supplemental calcium was provided, the animals were placed in an 0.8% solution of $CaCl_2$. The manner in which the calcium was assimilated from the solution will be considered later.

Roentgenograms of the frogs were made at the beginning of each experiment and at intervals thereafter. It was necessary to anesthetize the frogs (Rana pipiens) with ether before making the exposure; however, the toads (Xenopus laevis) could be kept quiet without anesthesia. Approximately 5 to 10 cc. of air was injected into the coelomic cavity to provide increased contrast in the roentgenograms. When great detail was desirable, ordinary x-ray film was found to be too coarse; a very fine grain emulsion was required. For this purpose, Kodak spectroscopic plates (Eastman Kodak Company), using the method of Tirman and Banker,[17] were satisfactory.[18]

Adequate blood samples be procured only by anesthetizing the frog with ether and then exposing and incising the heart, which bled directly into a small test tube. Blood serum inorganic phosphate was determined using the method of Shinowara and co-workers.[19] Serum calcium levels were obtained by the Clark and Collip modification of the Tisdall method.[20] To get sufficient quantities for these analyses it was necessary to pool the serum of two or three frogs.

Closed fractures were produced by breaking the left femur between thumb and forefinger.

RELATION OF LIME SACS TO ENVIRONMENTAL CALCIUM

The function of the lime sacs has never been clearly demonstrated. Schnitzer [21] stated that the size of the sacs has no relation to the season of the year, sex, cycle of sexual activity, or nutritional state. Krause suggested that the sacs might be a storage depot for excess calcium absorbed through the skin when the animal lived in calcium-rich water.[22] To prove this, he placed frogs in water having different concentrations of calcium chloride or strontium chloride and found that the sacs

16. Crystalline vitamin D_3 was obtained through the courtesy of Dr. M. L. Tainter, Sterling-Winthrop Research Institute, Rensselaer, N. Y.

17. Tirman, W., and Banker, H. W.: A Simple Method of Microradiography Using Ordinary Diagnostic X-Ray Equipment, Science **113**:530-531, 1951.

18. We are indebted to members of the Department of Radiology, Ohio State University Hospital, for the preparation of over 100 roentgenograms.

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22. Krause, D. K.: Experimentelle Untersuchungen über die Funktion der Kalksäckchen bei Froschlurchen, Ztschr. vergl. Physiol. **22**:346-358, 1935.

became filled with calcium or strontium carbonates. In 1942, Sulze [23] confirmed these results. Both Krause and Sulze stated that the calcium chloride was absorbed through the skin together with the water.

To serve as a control for subsequent studies on the effect of vitamin D or of fracture on the calcium deposits in the lime sacs, untreated frogs were placed in an

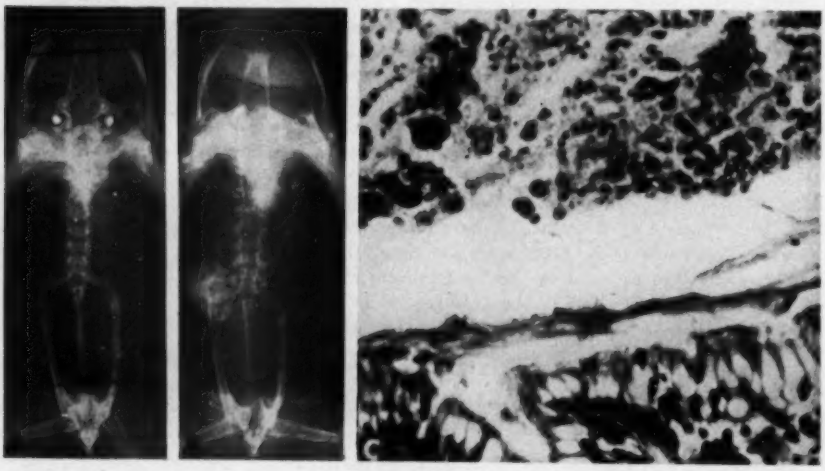

Fig. 4.—*A*, roentgenogram taken immediately before frog was placed in 0.8% $CaCl_2$; lime sacs scarcely visable. *B*, same frog after 22 days in $CaCl_2$; lime sacs moderately well filled; a mass of calcium salts is in the gut. *C*, calcospherites, some showing concentric lamellae, in lumen of gut. Hematoxylin and eosin; × 350.

TABLE 1.—*Effect of $CaCl_2$ and Vitamin D_2 on Lime Sacs and Bone**

Frog No.	Lime Sacs Before Treatment	Lime Sacs After 2 Wk. in $CaCl_2$	Dose of Vitamin D_2, Units	Days After Vitamin D_2	Lime Sacs After Vitamin D_2	Renal Calcif.	Ca in Gut	Osteoporosis
M	0	+	100,000	10	++	+	0	0
G	+	++	100,000	12	++++	+	0	+
X	0	+	100,000	13	+++	+++	0	+
A	+	++	100,000	15	++++	++	0	+
N	+	++	150,000	17	++++	++	0	+
L	0	+	150,000	20	+++	+++	0	+
P	0	+	150,000	20	+++	++++	0	+
T	0	0	150,000	21	+++	0	0	+
I-4	+	++	150,000	21	++++	+	0	+

* After initial roentgenograms were made, the frogs were placed in 0.8% $CaCl_2$; roentgenography was repeated two weeks later, and the first weekly intraperitoneal injection of 50,000 units of vitamin D_2 was given.

0.8% solution of calcium chloride. Roentgenograms taken at the beginning of the experiment showed very poor visualization of the lime sacs and no calcium in the intestine (Fig. 4*A*). After three weeks there was definite filling of the sacs (Fig. 4*B*; Tables 1 and 2). In several instances opacities appeared in the lumen

23. Sulze, W.: Über die physiologische Bedeutung des Kalksäckchenapparates bei Amphibien, Arch. ges. Physiol. **246**:250-257, 1942.

of the gut (Fig. 4*B* and *C*); subsequent histochemical examination proved them to be calcium phosphate and calcium carbonate. Metastatic calcification of tissues did not occur.

Although it seemed clear that calcium was entering the body of the frog, the portal of entry was unknown. The assumption that calcium can pass through the intact skin is untenable. Huf and Wills [24] have shown that, although isolated frog skin can take up sodium chloride against a concentration gradient, calcium does not move in the direction of the sodium ion and the chlorine ion. An oral route was denied by the statement of several investigators that the frog does not drink water.[25] However, using the method employed by Frank and Allee [26] in their study of fishes, it was found that after a frog had been kept for 24 hours in 0.1% colloidal thorium dioxide in distilled water, roentgenograms showed the colloid in the gastrointestinal

TABLE 2.—*Effect of $CaCl_2$ and Subsequent Fracture on Lime Sacs and Bone**

Frog No.	Lime Sacs Before Treatment	Lime Sacs After 4 Wk. in $CaCl_2$	Lime Sacs After Fracture	Age of Fracture, Days	Renal Calcif.	Ca in Gut	Osteoporosis	Fracture
II-10	+	++	+	9	0	0	0	Cartilagenous callus
II-9	+	++	++	11	0	0	0	Cartilagenous callus; periosteal bone
II-12	+	++	+	12	0	0	0	Cartilagenous callus
II-15	+	++	+	13	0	+	0	Cartilagenous callus; slight periosteal bone
II-18	±	++	+	13	0	0	0	Cartilagenous callus; slight periosteal bone
II-26	+	++	+	15	0	+	+	Cartilagenous callus; moderate periosteal bone
II-28	+	++	+	18	0	0	0	Cartilagenous callus; periosteal bone
II-25	±	++	+	50	0	0	0	Cartilagenous callus with calcification; periosteal bone
II-21	+	++	+	55	0	+	0	Cartilagenous callus with calcification; periosteal bone

* Frogs in 0.8% $CaCl_2$ for four weeks before fracture of left femur; thereafter in tap water.

tract. Because the frogs' skin is impermeable to colloids, the conclusion appears justified that the thorium dioxide was swallowed. This, then, is the manner in which frogs may obtain calcium from their aquatic environment.

RESPONSE OF LIME SACS TO VITAMIN D

Intramuscular injections of vitamin D_2 produced an accumulation of $CaCO_3$ in the lime sacs when 30,000 or more U. S. P. units were given without supplemental calcium (Table 3). Usually two to three weeks elapsed before a definitely increased

24. Huf, E. G., and Wills, J.: Influence of Some Inorganic Cations on Active Salt and Water Uptake by Isolated Frog Skin, Am. J. Physiol. **167**:255-260, 1951.

25. Overton, E.: Neununddreissig Thesen über die Wasserökonomie der Amphibien und die osmotischen Eigenschaften der Amphibienhaut, Verhandl. phys.-med. Gesellsch. **36**:277-295, 1904. Krogh, A.: Osmotic Regulation in Aquatic Animals, New York, The Macmillan Company, 1939. Adolph, E. F.: Physiological Regulations, Lancaster, Pa., Jaques Cattell Press, 1943.

26. Frank, P., and Allee, W. C.: Ingestion of Colloidal Thorium Dioxide by Representative Minnows from the Chicago Region, Physiol. Zool. **23**:134-139, 1950.

density of the sacs was evident in the roentgenograms. In animals surviving only a week or less after injection of the vitamin, even 50,000 units failed to produce a demonstrable change (Table 4). With higher doses of the vitamin and a longer time interval, the accumulation of calcium in the lime sacs was pronounced (Fig. 5; Table 4). In these frogs, kept in tap water, the calcium was obtained from the bones.

TABLE 3.—*Effect of Vitamin D_2 on Lime Sacs**

Frog No.	Lime Sacs Before Vitamin D	Dose of Vitamin D_2 Units	Lime Sacs 2 Wk. After Vitamin D	Lime Sacs 4 Wk. After Vitamin D	Comment
V-1	+	10,000	+	+	.....
V-2	+	10,000	+	+	.....
V-3	+	10,000	+	+	Unchanged in next 2 mo.
V-4	±	20,000	+	+	Unchanged in next 2 mo.
V-5	+	20,000	+	+	.....
V-6	+	20,000	+	+	.....
V-7	±	30,000	+	++	Slight cont'd. filling in next 2 mo.
V-9	±	30,000	+	++	Cont'd. filling in next 2 wk.
V-10	0	40,000	+	..	Frog died 3 wk. after vitamin injection
V-13	+	40,000	++	++	No change in next 2 mo.
V-14	+	40,000	++	+++	Cont'd. filling in next 2 mo.

* Frogs kept in tap water. Initial roentgenograms taken on day of the single intramuscular injection of vitamin D_2.

TABLE 4.—*Effect of Vitamin D_2 Given at Time of Fracture and During Healing**

Frog No.	Vitamin D_2 Total Units	Survival Period, Wk.	Lime Sacs	Renal Calcif.	Ca in Gut	Osteoporosis	Fracture
F-7	50,000	6	0	0	0	0	Hemorrhages and inflammation
F-18	50,000	6	0	0	0	0	Connective tissue callus; early cartilage
F-8	50,000	7	0	0	0	0	Connective tissue callus; early cartilage
F-9	50,000	8	0	0	0	0	Connective tissue callus; early cartilage
F-6	100,000	15	++	++	0	+	Cartilagenous callus; periosteal bone
F-5	100,000	16	0	0	0	0	Cartilagenous callus; extensive periosteal bone
F-3	200,000	27	+	0	0	++	Cartilagenous callus; slight periosteal bone
F-8	250,000	30	++	+	0	0	Calcif. in cartilagenous callus; periosteal bone
F-9	300,000	43	++	+++	0	+	Perivasc. calcif. in cartilagenous callus
F-2	500,000	74	++++	++	0	+++	Calcif. in cartilagenous callus; periosteal bone

* After fracture of the left femur each frog was given an intramuscular injection of 50,000 units vitamin D_2. Injection of the vitamin was repeated at weekly intervals; frogs kept in tap water.

Frogs previously kept in 0.8% $CaCl_2$ or placed in the solution at the time that administration of the vitamin was begun showed a remarkable accumulation of calcium in the sacs (Fig. 6; Table 1). This deposition of calcium often continued for many weeks after the last injection of the vitamin had been given (Fig. 7).

Histologically, the cuboidal epithelium of the sacs became flattened, often to such a degree that recognition of the cells was difficult. The spaces were filled with orthorhombic doubly refractile crystals of calcium carbonate (Fig. 3). The identification of these crystals as aragonite by means of x-ray diffraction was done for us by Mr. William L. Larsen, Department of Metallurgy, Ohio State University. This indicates that the chemical nature and molecular structure of the deposit in the lime sacs were not altered by vitamin D_2.

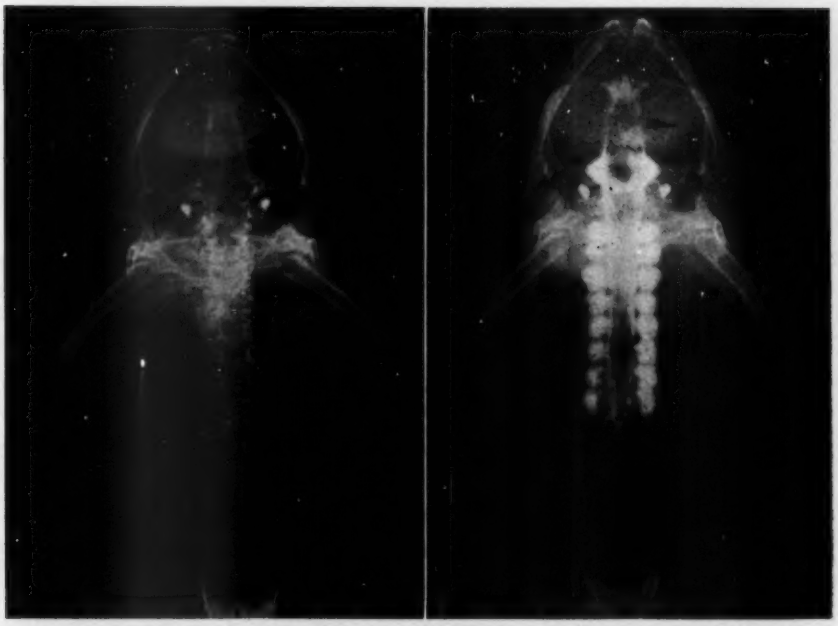

Fig. 5.—*A*, normal frog; note sharp outline of calvarium (arrow). *B*, same animal a week after the last of six weekly injections of 50,000 units of vitamin D. Lime sacs and endolymphatic extension about base of brain are filled with $CaCO_3$. Marked osteoporosis evidenced by poor outline of calvarium (arrow), diminished density of long bone cortices, and partial angulation of left humerus.

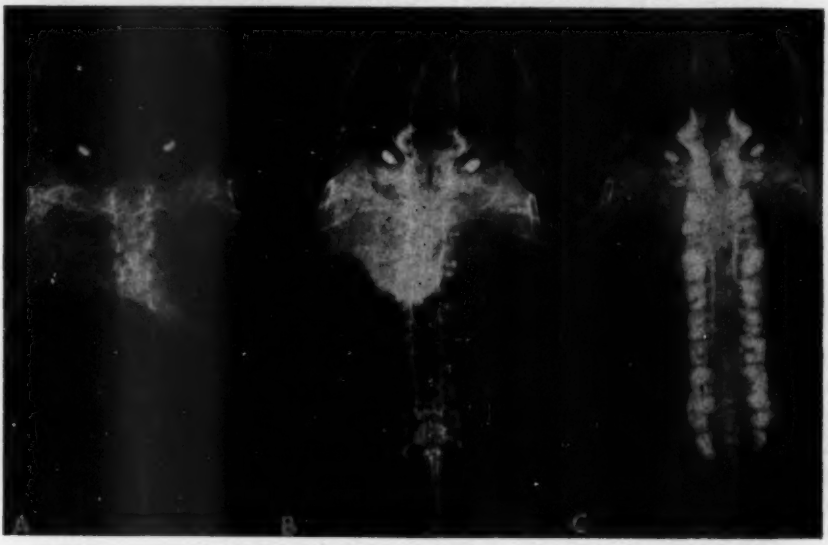

Fig. 6.—*A*, normal frog; lime sacs barely visable. *B*, same animal, after 18 days in 0.8% $CaCl_2$, showing partial filling of the lime sacs. *C*, same frog one week after the last of three weekly injections of 50,000 units of vitamin D; frog kept in 0.8% $CaCl_2$. Rapid filling of the lime sacs.

While these changes were taking place in the lime sacs, no demonstrable alteration in the size or density of the otoliths was noted. This is of interest because the otoliths are formed in a part of the labyrinth which is embryologically continuous with the endolymphatic extension that gives rise to the lime sacs.[11] The otoliths are the only other structures in vertebrates that are composed chiefly of $CaCO_3$. In the frog, the otoliths, like the crystals in the lime sacs, are of aragonite.[14a]

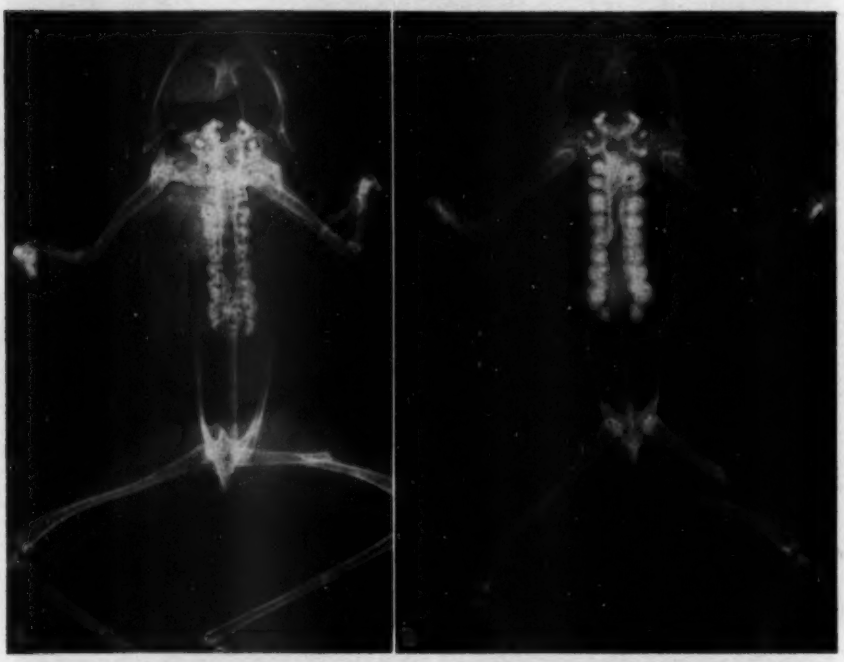

Fig. 7.—*A*, frog two weeks after the last of three weekly injections of 50,000 units of vitamin D; frog kept in 0.8% $CaCl_2$. Left femur fractured on day the roentgenogram was made. *B*, same frog 67 days later, kept in tap water and without additional vitamin D or food. Note continued filling of the lime sacs, calcification of the callus, and osteoporosis as shown by diminished density of bone cortices.

RESPONSE OF BONES TO VITAMIN D

When large doses of vitamin D are administered to frogs, with or without supplemental calcium available as $CaCl_2$ in the surrounding water, extensive osteoporosis occurs (Fig. 5; Tables 1, 4, and 5). Occasionally after an interval of two to three months, focal destruction of cortical bone may have progressed to the point where it has a cystic appearance in the roentgenogram (Fig. 8). As in cats [7] and rats,[27] so also in the frog these changes are most marked in the metaphyses of the long bones, particularly the femur and the tibia and fibula.

27. Carttar, M. S.; McLean, F. C., and Urist, M. R.: The Effect of the Calcium and Phosphorus Content of the Diet upon the Formation and Structure of Bone, Am. J. Path., **26**:307-331, 1950.

TABLE 5.—*Effect of Vitamin D_2 and $CaCl_2$ Given During Healing of Fracture**

Frog No.	Age of Fracture, Days	Vitamin D, Total Units	Lime Sacs	Renal Calcif.	Ca in Gut	Osteoporosis	Fracture
F-23	70	150,000	+++	+	+	0	Calcif. in cartilage; periosteal bone
F-24	22	150,000	+++	±	0	+	Cartilagenous callus; periosteal bone
F-22	23	150,000	++	0	+	0	Calcif. in cartilage; periosteal bone
F-31	36	250,000	+++	0	0	0	Calcif. in cartilage; periosteal bone
F-28	43	300,000	++++	±	+	0	Calcif. and bone in cartilage; periosteal bone
F-21	44	300,000	++++	++++	+	+	Calcif. about vessels; periosteal bone
F-26	45	300,000	++++	++++	0	++	Calcif.; periosteal bone
F-30	45	300,000	++++	+	+	+	Calcif.; periosteal bone
F-29	51	350,000	++++	++++	+	++	Calcif.; periosteal bone
F-27	58	400,000	++++	+++	0	++	Calcif.; periosteal bone

* Femur fractured and each frog given an intramuscular injection of 50,000 units of vitamin D_2 and placed in 0.8% $CaCl_2$. Vitamin D_2 injection repeated at weekly intervals.

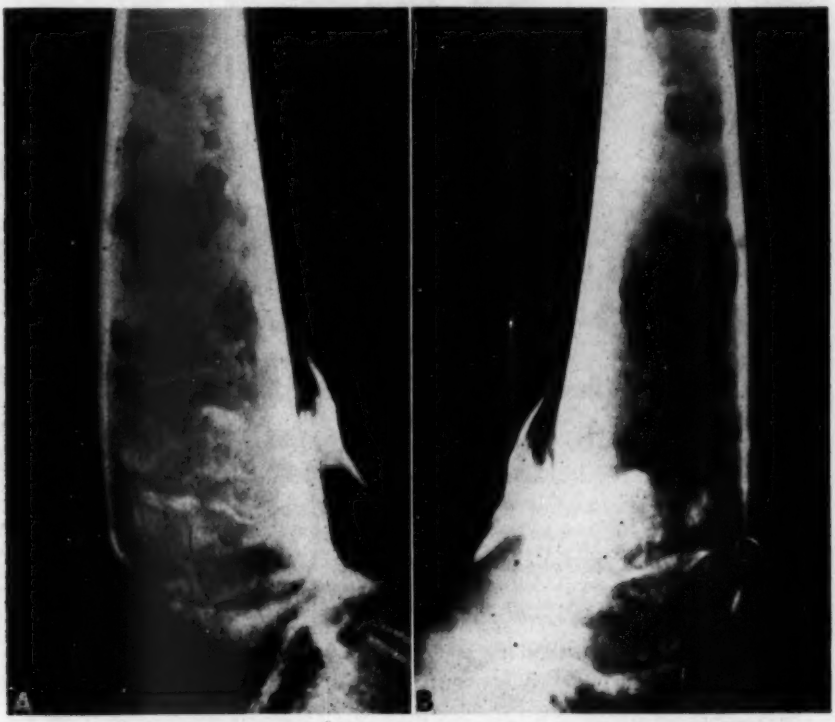

Fig. 8.—*A* and *B*, focal osteoporosis giving cystic appearance to the distal end of each femur. The changes first appeared 57 days after a single injection of 40,000 units of vitamin D. These roentgenograms on spectroscopic film were taken 33 days later; at that time the lime sacs showed good filling; × 15.

The occurrence of osteoporosis, in spite of supplemental calcium in the water, may be due to the absence of phosphorus. As emphasized by Carttar and co-workers,[27] what little phosphorus may be present in a high-calcium diet is converted in the intestinal tract into insoluble calcium phosphate. The importance of phosphorus for the deposition of bone salts is emphasized by the continued filling of the lime sacs with $CaCO_3$ under these same conditions.

Amphibian bone has a chemical composition [28] and histological structure [29] similar to that of mammalian bone. Trabeculae of cancellous bone, however, are not so

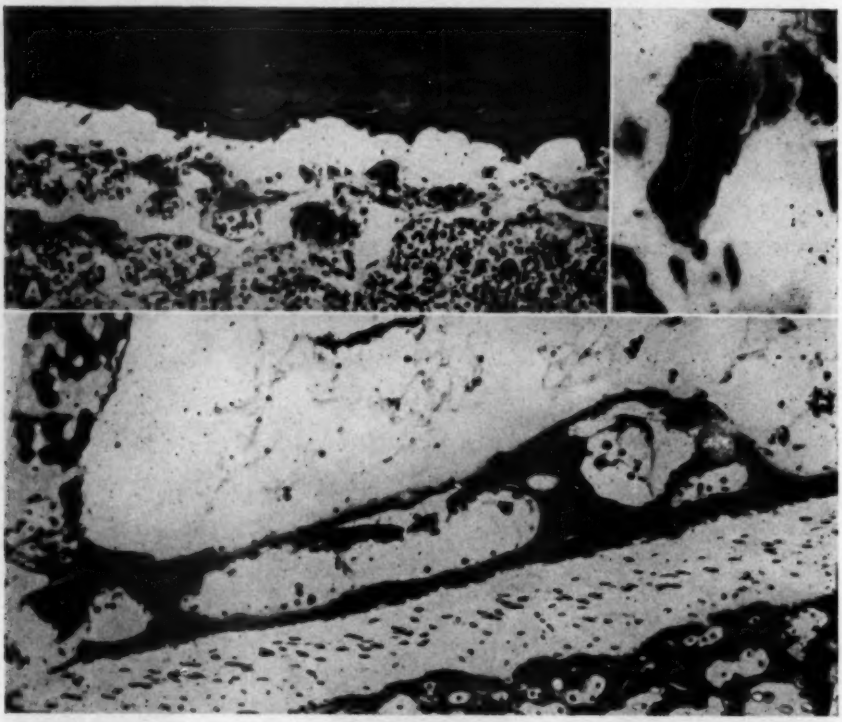

Fig. 9.—*A*, area of osteoporosis in femur showing numerous osteoclasts adjacent to Howship's lacunae in the overlying cortical bone. Frog has been kept in 0.8% $CaCl_2$ and received 100,000 units of vitamin D. Hematoxylin and eosin; × 200. *B*, one of the osteoclasts in Figure 9*A*; × 700. *C*, marked osteoporosis of cortical bone in proximal end of femur; frog kept in 0.8% $CaCl_2$ and received 150,000 units of vitamin D. A narrow band of decalcification lines the largest cyst; osteoclasts are not numerous. Hematoxylin and eosin; × 135.

prominent as they are in the long bones of birds and mammals; in Rana pipiens, used in these experiments, they are entirely absent. One of the outstanding features

28. Morgulis, S.: Studies on the Chemical Composition of Bone Ash, J. Biol. Chem. **93**:455-466, 1931.

29. Froböse, H.: Der Aufbau der Skeletteile in den freien Gliedmassen der anuren Amphibien, Morphol. Jahrb. **58**:473-566, 1927. Tretjakoff, D.: Die endochondrale Knochenbildung und die Verkalkung des Knorpels beim Frosch, Ztschr. Zellforsch. u. mikr. Anat. **9**:83-105, 1929.

of osteoporosis in man and other mammals, namely, the thinning and resorption of the trabeculae,[30] is therefore absent in the frog. The cortex of the amphibian bone, however, shows these same changes. Bone destruction is often associated with an increase in the number and activity of the osteoclasts and with the formation of Howship's lacunae (Fig. 9*A* and *B*). Osteoclastic proliferation has also been described accompanying bone destruction in hypervitaminosis D of guinea pigs.[2b]

In other areas early bone resorption appears to proceed without the intervention of osteoclasts. This is indicated by demineralization of the tissue and the appearance of a brush border of collagen fibers at its free margin (Fig. 9*C*). Similar changes during bone resorption have been described in mammalian bones and interpreted as indicating that the calcium is contained chiefly in the mucopolysaccharide cement substance which dissolves away before the collagenous fibers are destroyed.[31] This does not imply that osteoporosis in the frog is simply a process of demineralization

TABLE 6.—*Effect of Fracture on Lime Sacs and Bone**

Frog No.	Lime Sacs Before Fracture	Lime Sacs After Fracture	Age of Fracture, Days	Renal Calcif.	Ca in Gut	Fracture Repair
F-12	++	+	7	0	0	Hemorrhage; early periosteal reaction
F-14	+	±	12	0	0	Massive cartilagenous callus; no calcif. or bone
F-13	+	±	12	0	0	Massive cartilagenous callus; no calcif.; early periosteal bone
F-19	+	±	12	0	0	Cartilagenous callus; no calcif.; early periosteal bone
F-18	++	+	21	0	0	Cartilagenous callus; periosteal bone
F-13	0	0	30	0	0	Cartilagenous callus; slight periosteal bone; osteoporosis
F-16	+	0	42	0	0	Cartilagenous callus; perivasc. calcif.; periosteal bone
F-17	0	0	49	0	0	Cartilagenous callus; periosteal bone
F-39	0	0	49	0	0	Cartilagenous callus; perivasc. bone; periosteal bone

* Frogs kept in tap water before and after fracture. No vitamin D administered.

or halisteresis; the latter may slightly precede destruction of the organic matrix, but the two changes are essential to produce the lesion identified as "osteoporosis" in mammals.[32]

RELATION OF VITAMIN D AND CALCIUM DEPOT TO HEALING FRACTURES

Within three to five days of fracture the innermost layer of periosteal cells 1 to 2 mm. from the fracture site enlarge and appear as osteoblasts. Peripheral to these, the periosteal connective tissue proliferates to form a blastema which within a week has undergone extensive metaplasia and formed a massive cartilagenous callus. This primary cartilagenous callus is characteristic of amphibians, in contrast to mammals in which it plays a less prominent role.[33] Although periosteal bone formation may

30. (*a*) Mulligan, R. M.: Metastatic Calcification Associated with Hypervitaminosis D and Haliphagia, Am. J. Path. **22**:1293-1305, 1946. (*b*) Mulligan and Stricker.[4b] (*c*) Bauer.[7]

31. Ham, A. W.: Some Histophysiological Problems Peculiar to Calcified Tissues, J. Bone & Joint Surg. **34A**:701-728, 1952.

32. Footnote 1*b*. Footnote 27.

33. (*a*) Wurmbach, H.: Histologische Untersuchungen über die Heilung von Knochenbrüchen bei Amphibien, Ztschr. wissensch. Zool. **129**:253-358, 1927. (*b*) Pritchard, J. J., and Ruzicka, A. J.: Comparison of Fracture Repair in the Frog, Lizard, and Rat, J. Anat. **84**:236-261, 1950.

be extensive after two weeks, calcification of the cartilagenous callus is very slow and largely perivascular. Pritchard and Razicka [33b] have suggested that this may be due to the low phosphatase activity that they demonstrated in the cartilage of the frog. The cartilage is gradually replaced by bone through direct metaplasia of the cartilage and by bone formation in perivascular connective tissue.

The poor calcification of the cartilagenous callus and its slow replacement by bone calls for relatively small amounts of calcium. This is reflected in the slight depletion of the calcium deposits in the lime sacs produced by a healing fracture of the femur (Tables 2 and 6). Large doses of vitamin D had little, if any, demonstrable effect on the size of the callus or rate of calcification (Fig. 7; Table 4); in this, the response of the frog differs from that of the rat.[34] Osteoclastic resorption of the periosteal bone already laid down was occasionally observed at the fracture site in frogs on high doses of vitamin D. Frogs kept in 0.8% $CaCl_2$ and receiving massive doses of vitamin D (Table 5) showed earlier, though patchy, calcification of the cartilagenous callus. However, this was more closely comparable to the metastatic calcification of soft tissues in hypervitaminosis D than to any increased rate of fracture repair.

METASTATIC CALCIFICATION

One of the outstanding characteristics of hypervitaminosis D in man and animals is calcification of tissues that are normally uncalcified.[35] Chief among these are the kidneys, heart, lungs, and gastric mucosa. In the frog the most frequent site of metastatic calcification is in the kidneys; rarely are any other organs or tissues involved.

Nephrocalcinosis occurred when 100,000 units or more of vitamin D_2 were administered without supplemental calcium (Table 4). However, calcification was usually much more extensive when the animals were kept in a solution of 0.8% $CaCl_2$ while the vitamin was being absorbed (Tables 1 and 5). The calcium was largely in the lumen of the tubules (Fig. 10*A*); seldom was it seen in the epithelial cells. This may be attributed in part to the fact that substances which, like calcium, are in part resorbed by the tubules do not accumulate in the tubular epithelium but rather, as Smith [36] says, "are whisked through with lightning-like rapidity." The presence of calcium in the lumen of the renal tubules and its infrequent occurrence in their lixing cells resemble the condition seen in the lime sacs. In those sites accumulation of calcium in the lumen is often associated with a pronounced flattening of the epithelial cells.

The calcium in the kidney was present in all sections of the nephron but was most abundant in the proximal convoluted tubules. This is probably due to the alkaline reaction of the urine in the proximal convoluted tubule, for Montgomery and Pierce [37] have shown that in amphibians acidification takes place exclusively in the distal tubule. The reabsorption of phosphate occurs in the proximal tubule,[38] but

34. Copp, D. H., and Greenberg, D. M.: Studies on Bone Fracture Healing: Effect of Vitamins A and D, J. Nutrition **29**:261-267, 1945.

35. Footnote 1. Footnote 2*a*. Footnote 4*b*. Footnote 30.

36. Smith, H. W.: The Kidney: Structure and Function in Health and Disease, New York, Oxford University Press, 1951.

37. Montgomery, H., and Pierce, J. A.: The Site of Acidification of the Urine Within the Renal Tubule in Amphibia, Am. J. Physiol. **118**:144-152, 1937.

38. Walker, A. M., and Hudson, C. L.: The Rôle of the Tubule in the Excretion of Inorganic Phosphates by the Amphibian Kidney, Am. J. Physiol. **118**:167-173, 1937.

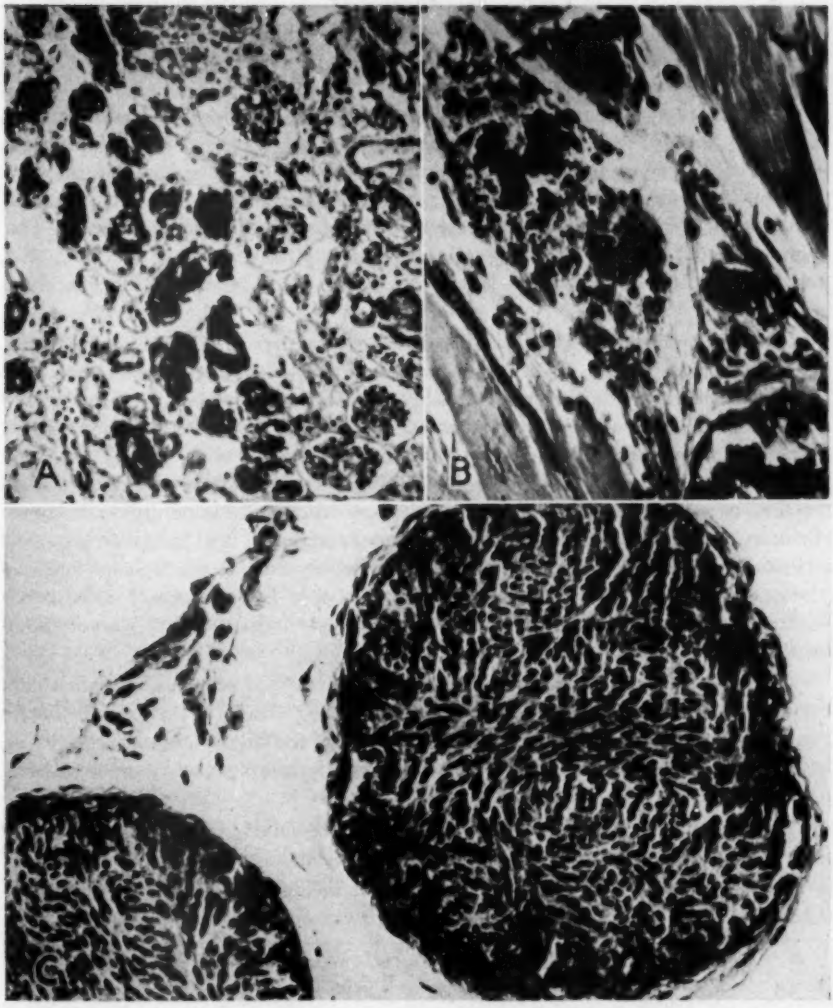

Fig. 10.—*A*, calcium phosphate in renal tubules. Frog had received 400,000 units of vitamin D but no $CaCl_2$ Hematoxylin and eosin; × 250. *B*, calcification in thigh muscle at site of vitamin injection. Frog kept in 0.8% $CaCl_2$ had received 150,000 units of vitamin D. Hematoxylin and eosin; × 300. *C*, normal parathyroids of specimen of Rana pipiens. Cells are elongated, have oval nuclei, and appear closely packed in broad interlacing bundles. Capillaries are limited to the capsule where they appear dark because filled with large nucleated erythrocytes. Hematoxylin and eosin; × 290.

this may be depressed by the vitamin D,[39] resulting in the presence of adequate amounts of phosphate in the proximal tubule to form the insoluble calcium phosphate that is precipitated at the alkaline pH of this segment. The chemical identity of the compound was determined in the histological section by using the molybdenum blue reaction for phosphate and alizarin red S for calcium, as described by Bunting.[40] The two reactions were positive.

It is deserving of emphasis that, despite filling of the lime sacs with calcium carbonate, areas of metastatic tissue calcification were composed of calcium phosphate. This difference in chemical composition between the salts stored in the lime sacs and those formed in areas of nephrocalcinosis may offer a partial explanation for the failure of the lime sacs to reduce metastatic calcification in the kidneys. The degree of renal calcification in specimens of Rana pipiens was not less than that found in specimens of Xenopus laevis, yet in the latter functional lime sacs are absent.

Calcification in the lungs was observed twice; it occurred in the connective tissue of the largest trabeculae. In the wall of the stomach a deposition of calcium was found only once; it was present as small masses in the submucosa. It is generally accepted that, because acids are eliminated by the lungs and gastric mucosa, these tissues are more alkaline, and therefore calcium deposition is favored.[30a] To account for its absence in the frog it might be argued that, because respiration occurs through the skin, as well as the lungs,[41] the latter do not develop the same degree of tissue alkalinity as is found in the lungs of mammals. Secretion of acid by the frog gastric mucosa is variable, being almost absent in the winter and, in the isolated mucosa, wholly dependent on external stimulation, for example, by histamine.[42] It is therefore possible that acid secretion was very low in these frogs, for they were without food during the experiment.

Although the endocardium and myocardium are frequent sites of metastatic calcification of mammals,[43] it was not observed in the frog. The effect of vitamin D_2 in raising the oxygen consumption of skeletal muscle in the frog [44] was not reflected in any morphological changes. In one instance there was a small deposit of calcium in the wall of the ductus arteriosus.

Areas of tissue damage, such as the site of vitamin D_2 injection in the thigh muscle (Fig. 10*B*), foci of liver necrosis due to infection, and degenerated parasites were often calcified. In this respect it is worth noting that following injection of 0.2 to 0.5 cc. of the vitamin in oil into the thigh numerous fat emboli were found in

39. Albright, F., and Reifenstein, E. C., Jr.: The Parathyroid Glands and Metabolic Bone Disease: Selected Studies, Baltimore, Williams & Wilkins Company, 1948.

40. Bunting, H.: Histochemical Analysis of Pathological Mineral Deposits at Various Sites, with Discussion of Methods Used, A. M. A. Arch. Path. **52**:458-469, 1951.

41. Dolk, H. E., and Postma, N.: Über die Haut- und die Lungenatmung von Rana temporaria, Ztschr. vergl. Physiol. **5**:417-444, 1927.

42. Crane, E. E., and Davies, R. E.: Observations on Some Physiological Variations in Frogs and Toads, with Special Reference to the Gastric Mucosa, Proc. Zool. Soc. London **120**:471-483, 1950.

43. Ham, A. W.: Mechanism of Calcification in the Heart and Aorta in Hypervitaminosis D, Arch. Path. **14**:613-626, 1932. Mulligan.[30a] Mulligan and Stricker.[45]

44. Gelfan, S.: Effect of Viosterol upon Oxygen Consumption of Frog's Muscle, Am. J. Physiol. **113**:464-466, 1935.

the capillaries of the glomeruli, lungs, and even the capsule of the parathyroids. However, there was no evidence of local tissue damage, and there was no calcium deposited in the immediate vicinity of the emboli.

Calcification of the intestinal wall was never seen, but large quantities of calcium were occasionally present in the lumen, where they were visible in the roentgenogram (Fig. 4*B*). Histological sections through these areas revealed large numbers of minute calcospherites (Fig. 4*C*). Unlike the areas of tissue calcification, which were calcium phospate, the calcium in the lumen of the gut was largely calcium carbonate. This was determined by placing a drop of dilute HCl on the section; the resultant gas was identified as carbon dioxide according to the method of Bunting.[40] The infrequent presence of large amounts of calcium in the intestinal tract of frogs kept in 0.8% $CaCl_2$ is probably due to the effectiveness of the administered vitamin D in promoting calcium absorption.[39]

EFFECT OF VITAMIN D_2 ON PARATHYROID GLANDS

The relation of the parathyroids to calcium metabolism in the frog derives special interest from the fact that amphibians are the first animals in the phylogenetic scale that possess these organs.[45] In Rana pipiens they are four in number, two on each side, near the jugular body. They are translucent white bodies, 0.5 mm. in diameter, best studied by serial sections of the glands and neighboring tissue obtained by block dissection. The cells are oval with large chromatin rich nuclei; usually the cells are closely packed (Fig. 10*C*), but occasionally they may be separated and the cytoplasm appear vacuolated. This is a stage in their cyclic degeneration that begins in winter; regeneration is completed by June.[46] Blood vessels are absent from the parenchyma; however, capillaries are abundant in the capsule. Few experimental studies have been carried out on the parathyroids of adult frogs; Waggener [47] was able to induce tetany in bull frogs, Rana catesbeiana, by removal of the glands. He observed a fall in the blood calcium level from an average of 11.9 mg. to 7.7 mg. per 100 cc.

Although hypervitaminosis D in dogs is accompanied by decrease in size of the parathyroids and degenerative changes in their cells,[4b] no clear evidence of such changes could be observed in the frog. Numerous micrometric measurements of sections of the parathyroids failed to show any consistent alteration in their size. In view of the cyclic changes that normally occur in the parathyroid of the frog, no distinct histologic changes could be attributed to the action of vitamin D_2.

As a corollary to these studies of the effect of vitamin D_2 on the amphibian parathyroid, a preliminary investigation of the action of mammalian parathyroid injection (Paroidin) on the frog was carried out. Ten leopard frogs received 10 units of parathyroid injection intraperitoneally twice daily for 20 to 25 days. At the end of

45. Bargmann, W.: Die Epithelkörperchen, in Handbuch der mikroskopischen Anatomie des Menschen, edited by W. von Mollendorff, Berlin, Springer-Verlag, 1939, Vol. 6, Pt. 2, pp. 137-196.

46. Romeis, B.: Morphologische und experimentelle Studien über die Epithelkörperchen der Amphibien: Die Morphologie der Epithelkörper der Anuren, Ztschr. Anat. **80**:547-578, 1926. Waggener, R. A.: A Histological Study of the Parathyroids in the Anura, J. Morphol. **48**:1-43, 1928.

47. Waggener, R. A.: An Experimental Study of the Parathyroids in the Anura, J. Exper. Zool. **57**:13-55, 1930.

this time there was a slight increase in the amount of calcium in the lime sacs of only six frogs. There was no nephrocalcinosis, and the bones showed only an occasional small focus of osteoclast activity.

EFFECT OF VITAMINS D_2 AND D_3 AND OF CALCIUM CHLORIDE ON SERUM CALCIUM AND PHOSPHATE LEVELS

The manner of collecting and analyzing the serum for calcium and phosphate has been described in the section on methods.[48] No significant changes in the serum calcium and phosphate levels were observed when leopard frogs were treated with $CaCl_2$, vitamin D_2, or vitamin D_3; however, combined treatment with $CaCl_2$ and vitamin D_2 led to a significant elevation of the calcium level (Table 7). The same combination also resulted in the most rapid filling of the lime sacs. This suggests that the lime sacs may act as a reservoir for excess calcium, maintaining the serum calcium at a fairly constant level.

In the rat, Morgan and co-workers [49] have shown that vitamin D_2 (irradiated ergosterol) is twice as toxic as vitamin D_3 (activated 7-dehydrocholesterol). The

TABLE 7.—*Effect of Vitamin D and $CaCl_2$ on Serum Calcium and Phosphate**

Treatment	No. of Frogs	Av. Wt., Gm.	No. of Pooled Blood Samples	Ca, Av. Mg. per 100 Cc.	Phosphate, Av. Mg. per 100 Cc.
	Rana pipiens				
Control	24	35.0	12	8.7	5.6
$CaCl_2$	19	36.3	12	8.3	5.7
Vitamin D_2	24	38.5	11	9.1	6.8
Vitamin D_3	14	38.0	6	8.3	6.9
$CaCl_2$ and vitamin D_2	22	37.4	7	11.4	6.6
	Xenopus laevis				
Control	6	51.3	4	8.9	9.6
Vitamin D_2	6	50.1	4	11.4	9.5
Vitamin D_3	6	52.0	4	11.5	9.8

* Intramuscular injections of vitamin D_2 or D_3 were administered in doses of 2,500 units per gram of frog, repeated one week later at 5,000 units per gram. Frogs receiving $CaCl_2$ were kept in an 0.8% solution of the salt. Blood was collected one week after the second injection of vitamin D. All frogs were sexually mature females, but the specimens of Xenopus laevis were not full grown.

toxicity, however, correlates well with the relatively greater hypercalcemic effect of vitamin D_2.[50] In the frog, too, vitamin D_2 is more effective than vitamin D_3 in mobilizing calcium from the bones. In a group of 12 frogs, Rana pipiens, roentgenograms showed the lime sacs faintly (plus-minus) visible. Six were then treated with 100,000 units of vitamin D_2 and six with 100,000 units of vitamin D_3. Roentgenograms made three weeks later showed that in the frogs receiving vitamin D_2 the degree of filling of the sacs rated a 2 plus, whereas in those receiving vitamin D_3 the filling was distinctly less, rating only 1 plus.

48. Professor G. Shinowara supervised the technical procedures.

49. Morgan, A. F.; Kimmel, L., and Hawkins, N. C.: A Comparison of the Hypervitaminoses Induced by Irradiated Ergosterol and Fish Liver Oil Concentrate, J. Biol. Chem. **120**:85-102, 1937.

50. McChesney, E. W.: Chronic Toxicity and Hypercalcemic Effect of Various Activated Sterols in the Albino Rat, Proc. Soc. Exper. Biol. & Med. **57**:29-31, 1944. Mulligan and Stricker.[4b]

EFFECT OF VITAMIN D ON XENOPUS LAEVIS

The aquatic South African clawed toad, Xenopus laevis, belongs to one of the most "primitive" families (Pipidae) of the tailless amphibia.[51] In another primitive frog, Discoglossus pictus, Coggi[10c] found no vertebral extension of the endolymphatic duct. This, coupled with the report by Bruce and Parkes[52] of severe rickets and osteoporosis in young specimens of Xenopus laevis on diets low in calcium and vitamin D, suggested that in this species the lime sacs might be functionally or structurally different from those of Rana pipiens.

In three male and three female fullgrown specimens of Xenopus laevis, intramuscular injection of 250,000 units of vitamin D_2 failed, after the lapse of three weeks, to visualize any structures identifiable as lime sacs in the roentgenograms. The animals were then placed in 0.8% $CaCl_2$, but after three weeks lime sacs still could not be seen. Because vitamin D_3 is almost a thousand times more effective in chickens than is vitamin D_2,[53] the possibility of such a difference in the effectiveness of these two vitamins D in Xenopus laevis was considered. Four toads were given intramuscular injections of 1,000,000 units of vitamin D_3; the remaining two were given injections with 1,000,000 units of vitamin D_2. Four weeks later roentgenograms showed no lime sacs, although the average serum calcium level was 16.3 mg. per 100 cc., and that of phosphorus was 8.6 mg. per 100 cc. The average normal calcium level in the male Xenopus lavis is 7.6 mg. per 100 cc. and in the female, 9.8 mg. per 100 cc.[54]

Dissection of the treated toads failed to reveal any structures identifiable as paravertebral lime sacs. Histological sections through the vertebrae at the level of the nerve roots were likewise negative.

The only reference to lime sacs in the Xenopidae is by Dempster,[10a] who writes that "one specimen of Xenopus mulleri which was examined has an endolymphatic sac of the same structure and extent as that of Rana. . . . Specimens of Xenopus laevis on dissection showed crystalline accumulations in the endolymphatic sac." Whether Dempster meant to imply that Xenopus laevis also had sacs of the same extent as Rana pipiens is difficult to determine. Our findings do not support such a conclusion.

To ascertain the action of lime sacs in maintaining the normal serum calcium level, specimens of Rana pipiens and Xenopus laevis were given comparable amounts of vitamin D_2 or vitamin D_3. In specimens of Rana pipiens there was little change in the calcium level; in specimens of Xenopus laevis there was a significant elevation (Table 7).

The osteoporosis and metastatic calcification observed in African toads receiving very large amounts of vitamin D was not noticeably different in character or degree

51. Noble, G. K.: The Biology of the Amphibia, New York, McGraw-Hill Book Company, Inc., 1931.

52. Bruce, H. M., and Parkes, A. S.: Rickets and Osteoporosis in Xenopus laevis, J. Endocrinol. **7**:64-81, 1950.

53. Prosser, C. L., Editor: Comparative Animal Physiology, Philadelphia, W. B. Saunders Company, 1950.

54. Charles, E.: Metabolic Changes Associated with Pigmentary Effector Activity and Pituitary Removal in Xenopus Laevis: Calcium and Magnesium Content of the Serum, Proc. Roy. Soc., London, s.B **107**:504-510, 1931.

from that seen in specimens of Rana pipiens. Xenopus laevis resembles Rana pipiens in that no endochondral bone formation occurs in the shaft, but by the age of one year the epiphyseal cartilage of the toad has been irregularly eroded and calcified, forming trabeculae at the ends of the bone.[55] These trabeculae were almost completely resorbed in specimens of Xenopus laevis receiving high doses of vitamin D.

In view of the absence of functional lime sacs in Xenopus laevis, the possibility existed that other structures concerned with calcium metabolism, namely, the parathyroids, might be altered. We were never able to identify these glands in gross dissections but, by making serial sections, found them on the slides. In these preparations the parathyroids of Xenopus laevis were approximately equal in size and histologolically similar to those of Rana pipiens.

COMMENT

Large stores of calcium carbonate are rarely present in vertebrate animals; when encountered, they are nearly always within evaginations of the endolymphatic organ. Since this structure produces the otoliths, which also are composed of calcium carbonate, the evolution of calcium depots from that organ is comprehensible. Students of evolution would consider this a type of preadaptation.[56] The frog or its ancestors were preadapted by having available for development the otolith-forming endolymphatic organ.

During the Devonian period the crossopterygian fishes evolved into the lung fishes and the amphibians. It is therefore significant that in the African lungfish, Protopterus aethiopicus, diverticuli of the saccus endolymphaticus almost cover the fourth ventricle and reach caudad to the first sensory spinal nerve. They are lined by cuboidal epithelium and contain crystals of calcium carbonate.[57] In their extent and structure they resemble the condition found in the urodele amphibia.

In view of the known blood buffering action of the calcareous shells of some molluscs [58] and of the calciferous glands of the earthworm,[59] the possibility that the endolymphatic organs of the lungfish and amphibians have a similar function must be considered. Evidence in favor of this possibility was obtained by Sulze,[23] who observed the diminution or even complete disappearance of calcium from the lime sacs of frogs kept in tap water through which was bubbled a gentle stream of carbon dioxide.

In the next higher class of vertebrates, the reptiles, the endolymphatic duct occasionally extends onto the dorsum of the medulla, but nothing comparable to that in the frog is encountered.[10a] However, in several species of lizard the duct leaves

55. Fox, E., and Irving, J. T.: The Ossification Process in the Long Bones of Xenopus Lævis, South African J. M. Sc. **15**:5-10, 1950.

56. Carter, G. S.: Animal Evolution: A Study of Recent Views of Its Causes, London, Sidgwick & Jackson, Ltd., 1951.

57. Burckhardt, R.: Das Centralnervensystem von Protopterus annectens, Berlin, J. Sittenfeld, 1892.

58. Dotterweich, H., and Elssner, E.: Die Mobilisierung des Schalenkalkes für die Reaktionsregulation der Muscheln (Anodonta cygnea), Biol. Zentralbl. **55**:138-163, 1935.

59. Robertson, J. D.: The Function of the Calciferous Glands of Earthworms, J. Exper. Biol. **13**:279-297, 1936.

the skull and ends as a subcutaneous sac in the neck. Instances of abnormal enlargement and distention of these sacs by calcareous material have been reported.[60] Their normal function has not been investigated.

Although the calcium carbonate in the lime sacs of amphibia may serve as a source of carbonate in the blood buffering mechanism, its most important function is probably as a storage site for calcium. The absence of osseous trabeculae in the long bones of the frog is noteworthy. In other animals they serve as a ready source of calcium; its disappearance does not markedly weaken the shaft. Resorption of bone in Rana pipiens is at once manifest in the cortex, greatly impairing its strength.

The storage of calcium in the lime sacs not only makes available additional quantities of calcium for skeletal growth and repair but also removes from the circulation excess quantities of calcium that cannot be quickly excreted in the urine and feces. As shown in these experiments, high doses of vitamin D alone failed to produce a significant elevation of serum calcium in specimens of Rana pipiens. The calcium was deposited in the lime sacs almost as rapidly as it was liberated from the bones or absorbed from the gut. The effectiveness of these structures in maintaining a constant level of serum calcium despite the action of vitamin D is illustrated by the higher levels attained in the similarly treated specimens of Xenopus laevis, in which functional lime sacs could not be demonstrated.

The fact that storage of calcium carbonate occurs so rapidly in the lime sacs after administration of vitamin D raises the question whether this increase is due to a specific action of the vitamin upon the epithelium of the sacs. Such an effect of vitamin D is generally recognized in its enhancement of calcium absorption through the intestinal mucosa [39] and of phosphate by the renal tubular epithelium.[61] Nevertheless, against the assumption of a specific action on the lime sac epithelium is the fact that a large dose (30,000 units) is necessary to visibly increase storage in the sacs and that when storage is most rapid the animals are imbibing $CaCl_2$, which is quickly absorbed from the gut. It appears probable that vitamin D enhances filling of the sacs chiefly by bringing calcium into the circulation, from which it is rapidly and selectively removed by the lime sacs when the serum calcium level is elevated.

SUMMARY

Attention is directed to the existence of a potentially large calcium depot in the paravertebral lime sacs of the frog.

The lime sacs arise from the endolymphatic sac of the labyrinth. The calcium in the lime sacs, as well as that of the otoliths, is agragonite, a form of calcium carbonate.

In frogs kept in an 0.8% solution of calcium chloride the lime sacs become larger and more radio-opaque due to the accumulation in them of calcium carbonate.

Contrary to statements in the literature, frogs do swallow water; the calcium in the water is absorbed through the intestinal mucosa, not through the skin. In the absence of vitamin D, enough calcium may accumulate in the intestinal tract, particularly in the rectum, to be visible in a roentgenogram.

60. Mertens, R.: Über eine merkwürdige Erkrankung des Gehörorgans bei Anolis und anderen Eidechsen, Bl. Aquar. u. Terrarienk. **38**:13-14, 1927.

61. Klein, R., and Gow, R. C.: Interaction of Parathyroid Hormone and Vitamin D in the Renal Excretion of Phosphate, J. Clin. Endocrinol. **13**:271-282, 1953.

If vitamin D in amounts of 50,000 units or more is administered to frogs kept in 0.8% $CaCl_2$, there is no accumulation of calcium in the intestinal lumen, indicating rapid absorption by the intestinal mucosa. The lime sacs fill rapidly.

Vitamin D administered as intramuscular injections of 30,000 units or more will lead to deposition of calcium in the lime sacs, even when supplemental calcium is absent from the water.

When large amounts of vitamin D are administered, osteoporosis occurs, characterized by the resorption of cortical bone usually associated with proliferation of osteoclasts. This may occur even if supplemental calcium is present in the water.

Large doses of vitamin D had little effect on bone healing; occasionally there was resorption of newly formed periosteal bone in the region of the fracture.

Metastatic calcification occurred almost exclusively in the kidney. The lung and gastric mucosa were rarely involved.

Calcium was present in the kidneys as the phosphate, not as the carbonate salt, although the latter filled the lime sacs.

Excessive amounts of vitamin D_2 produced no morphologic changes in the parathyroids.

Vitamin D_2 is more potent in mobilizing calcium in the frog than is vitamin D_3.

No functional lime sacs could be demonstrated in the primitive African clawed toad, Xenopus laevis.

The role of the lime sacs in preserving a fairly constant serum calcium level is indicated by the normal levels maintained by specimens of Rana pipiens treated with large doses of vitamin D. The sacless specimens of Xenopus laevis showed a significant rise in serum calcium following administration of vitamin D.

The phylogenetic relationship of animals with calcium-containing extensions of the endolymphatic duct is reviewed.

Various functions of the paravertebral lime sacs are discussed.

Vitamin D probably has no specific action on the epithelium of the lime sacs.

EFFECT OF INJECTING RATS WITH HOMOLOGOUS RENAL TISSUE MIXED WITH ADJUVANTS OR STREPTOCOCCI

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THE ADJUVANT technique of Freund,[1] whereby antibody formation to certain antigens is enhanced, has been used to great advantage by Kabat, Wolf, and Bezer[2]; Morgan,[3] and Lipton and Freund[4] in the study of experimental encephalomyelitis, in which demyelinating lesions of the central nervous system are readily produced by the injection of homologous or heterologous brain tissue suspended in adjuvants. Frick[5] described lesions of interstitial myocarditis in rats given injections of homologous heart tissue in adjuvants, whereas Peck and Thomas[6] were unable to produce renal or cardiac lesions in rabbits given injections of homologous kidney or heart tissue in adjuvants.

Aided by grants from the Life Insurance Medical Research Fund and the Helen Hay Whitney Foundation.

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1. Freund, J.; Stern, E. R., and Pisansi, T. M.: Isoallergic Encephalomyelitis and Radiculitis in Guinea Pig After One Injection of Brain and Mycobacteria in Water-in-Oil Emulsion, J. Immunol. **57**:179, 1947.

2. Kabat, E. A.; Wolf, A., and Bezer, A. E.: The Rapid Production of Acute Disseminated Encephalomyelitis in Rhesus Monkeys by Injection of Heterologous and Homologous Brain Tissue with Adjuvants, J. Exper. Med. **85**:117, 1947; Studies on Acute Disseminated Encephalomyelitis Produced Experimentally in Rhesus Monkeys, ibid. **88**:417, 1948.

3. Morgan, I. M.: Allergic Encephalomyelitis in Monkeys in Response to Injection of Normal Monkey Nervous Tissue, J. Exper. Med. **85**:131, 1947.

4. Lipton, M. M., and Freund, J.: Encephalomyelitis in the Rat Following Intracutaneous Injection of Central Nervous System Tissue with Adjuvant, Proc. Soc. Exper. Biol. & Med. **81**:260, 1952.

5. Frick, E.: Tierexperimentelle Untersuchungen über eine tuberkulös-allergische Myokarditis, Ztschr. ges. exper. Med. **117**:393, 1951.

6. Peck, J. L., and Thomas, L.: Failure to Produce Lesions or Autoantibodies in Rabbits by Injecting Tissue Extracts, Streptococci and Adjuvants, Proc. Soc. Exper. Biol. & Med. **69**:451, 1948.

Studies made by the Caveltis[7] in 1945 indicated that lesions of glomerulonephritis could be produced in rats by the intraperitoneal injection of homologous renal tissue mixed with ether-killed Group A hemolytic streptococci. Their investigation also disclosed that antibodies to rat renal tissue developed in the experimental animals. Subsequent investigators, however, failed to confirm these observations.[8]

In the instances of experimental encephalomyelitis and glomerulonephritis, one explanation advanced to account for the development of lesions is that the adjuvants or streptococci enhance tissue antigenicity and potentiate isoantibody (or autoantibody) formation and that the in vivo interaction of the antibodies with the corresponding tissue induces the experimental disease.

The purpose of the present experiments was twofold: (1) to investigate the effect on rats of injecting homologous kidney mixed with Freund's adjuvants, and (2) to attempt to reproduce the experimental results described by Cavelti, namely, to produce nephritis by injecting rats with homologous kidney mixed with killed hemolytic streptococci.

Certain modifications of these two experiments were undertaken: (1) Some animals injected with kidney-adjuvant mixtures were also treated with desoxycorticosterone acetate and NaCl; (2) some rats injected with kidney-streptococcus mixtures were also given desoxycorticosterone acetate and NaCl, and (3) other groups of rats immunized with kidney-streptococcus mixtures received different serological types of Group A hemolytic streptococci with the kidney in successive courses of injections. These modifications were introduced for two reasons. The first is that desoxycorticosterone acetate and NaCl are known to enhance the severity of lesions of glomerulonephritis induced by injection of rabbit anti-rat-kidney serum in rats.[9] Secondly, the concept that repeated exposure to different serological types of streptococci may be an important factor in the development of experimental rheumatic-like cardiac lesions in rabbits[10] suggested the possibility that a similar influence might be exerted in experimental glomerulonephritis.

METHODS

The table indicates the disposition of the 103 animals used in the experiment. Long-Evans strain rats of both sexes, approximately 50 days old and weighing about 90 gm. at the beginning

7. (*a*) Cavelti, P. A., and Cavelti, E. S.: Studies on the Pathogenesis of Glomerulonephritis: I. Production of Autoantibodies to Kidney in Experimental Animals, Arch. Path. **39**:148, 1945; (*b*) Studies on the Pathogenesis of Glomerulonephritis: II. Production of Glomerulonephritis in Rats by Means of Autoantibodies to Kidney, ibid. **40**:158, 1945; (*c*) Studies on the Pathogenesis of Glomerulonephritis: III. Clinical and Pathologic Aspects of the Experimental Glomerulonephritis Produced in Rats by Means of Autoantibodies to Kidney, ibid. **40**:163, 1945.

8. Pyles, W. J.: Personal communication to the authors. Humphrey, J. H.: The Pathogenesis of Glomerulonephritis: A Reinvestigation of the Auto-Immunisation Hypothesis, J. Path. & Bact. **60**:211, 1948.

9. Knowlton, A. I.; Stoerk, H. C.; Seegal, B. C., and Loeb, E. N.: Influence of Adrenal Cortical Steroids upon the Blood Pressure and the Rate of Progression of Experimental Nephritis in Rats, Endocrinology **38**:315, 1946; Desoxycorticosterone Acetate: The Potentiation of Its Activity by Sodium Chloride, J. Exper. Med. **85**:187, 1947.

10. Murphy, G. E., and Swift, H. F.: Induction of Cardiac Lesions, Closely Resembling Those of Rheumatic Fever in Rabbits, Following Repeated Skin Infections with Group A Streptococci, J. Exper. Med. **89**:687, 1949; The Induction of Rheumatic-Like Cardiac Lesions in Rabbits by Repeated Focal Infections with Group A Streptococci: Comparison with the Cardiac Lesions of Serum Disease, ibid. **91**:485, 1950.

of the experiment, were used throughout. All rats were maintained on a diet of Rockland D-3 rat pellets, except those receiving injections of rat kidney and streptococci. The latter groups were fed a diet consisting of 60% Purina dog chow and 40% scratch, oats, bread, and fresh carrots.[7b] Animals treated with desoxycorticosterone acetate were supplied with 0.85% NaCl in the drinking water during the experiment.

Sterile perfused kidney, heart, and placenta of normal adult rats were obtained according to the method described by Smadel.[11] The tissue-adjuvant mixtures were prepared by thoroughly emulsifying in a Waring Blendor 18 gm. of the tissue, 20 ml. saline, 20 ml. molten Aquaphor, and 40 ml. paraffin oil containing 100 mg. heat-killed tubercle bacilli (Strain H37RV). These emulsions were then heated at 60 C. for 45 minutes and stored in the refrigerator, at 4 C. When adjuvant was used alone, the tissue was replaced by an equal volume of saline.

The streptococci used were all of Group A—Strain NY5 (type 10 or 12),[12] Strain C203 (type 3), and Gay strain (type 30). Virulence of the organisms was confirmed by repeated mouse passage. Strain NY5 was grown according to the method of Bernheimer;[13] Strain

Disposition of Animals: Pathological Findings

Group	No. of Animals	Type of Injection	Pathology
I A	16	3 to 4 series, 9 to 12 ml. kidney-adjuvant mixture	4 focal interstitial nephritis, 1 focal interstitial myocarditis
I B	9	4 injections of 0.5 to 1.0 ml. kidney-adjuvant mixture	1 pyelonephritis
I C*	15	2 to 3 series, 6 to 9 ml. kidney-adjuvant mixture	3 focal interstitial nephritis, 2 eosinophilic inflammation, 1 hydronephrosis
II A†	8	3 series, 9 ml. heart-adjuvant mixture	None
II B†	6	2 series, 3 ml. placenta-adjuvant mixture	1 focal interstitial nephritis, 1 eosinophilic inflammation
II C†	6	Desoxycorticosterone acetate, total dose 115 mg.	None
II D†	8	3 series, 9 ml. adjuvant without tissue	1 focal interstitial nephritis, 1 eosinophilic inflammation
III A	8	3 series, 45 ml. NY5-kidney mixture	1 focal interstitial nephritis, 2 eosinophilic inflammation
III B	6	4 series, 42 ml. NY5-kidney mixture, 12 ml. C203-kidney mixture, 10 ml. Gay strain-kidney mixture	1 focal interstitial nephritis, 2 intra-abdominal abscess
III C‡	14	2 series, 20 ml. NY5-kidney mixture	4 focal interstitial myocarditis
IV†	7	4 series, 32 ml. NY5 suspension, 10 ml. C203 suspension, 10 ml. Gay strain suspension	2 focal interstitial myocarditis, 1 periarteritis

* Received desoxycorticosterone acetate, total dose 115 mg., from 10 days before to 25 days after the first series.

† Control.

‡ One half of this group received desoxycorticosterone acetate, total dose 115 mg., from 6 days before to 28 days after first series. The remainder received same dose from 6 days before to 28 days after second series.

C203[14] was grown in a medium containing 4% tryptic-digested proteose peptone No. 3 (Difco), 3% glucose, 0.3% sodium bicarbonate, and other accessory factors, and the Gay strain was grown in Todd-Hewitt broth. The organisms were collected by centrifugation in a Sharples centrifuge; the wet volume of the packed organisms was determined, and a 2% suspension in saline was prepared. Ether was added to 10% by volume, which killed the organisms in 24 to 48 hours at 4 C. The suspensions were stored until needed in a cabinet refrigerated with solid CO_2.

11. Smadel, J. E.: Experimental Nephritis in Rats Induced by Injection of Anti-Kidney Serum: Preparation and Immunological Studies of Nephrotoxin, J. Exper. Med. **64**:921, 1936.

12. Watson, R. F., and Lancefield, R. C.: Studies on the Antigenic Composition of Group A Hemolytic Streptococci: Types with Serologically Identical M but Distinct T Antigens; Types 10 and 12, J. Exper. Med. **79**:89, 1944.

13. Bernheimer, A. W.; Gillman, W.; Hottle, G. A., and Pappenheimer, A. M., Jr.: An Improved Medium for the Cultivation of Hemolytic Streptococcus, J. Bact. **43**:495, 1942.

14. Supplied by Dr. Seymour A. Halbert.

Sterile rat kidney was homogenized in a Waring Blendor with enough isotonic saline to prepare a 20% suspension and was stored in the solid CO_2 cabinet. Mixtures for injection containing streptococci and tissue were prepared by combining equal volumes of the 2% streptococcus suspension and 20% tissue suspension under aseptic conditions.

Kidney-adjuvant mixtures were injected by the intramuscular or subcutaneous route in 1-ml. amounts and kidney-streptococcus mixtures in 1- or 2-ml. quantities by the intraperitoneal route. Each course consisted of three weekly injections of the kidney-adjuvant mixtures or 8 to 20 daily injection of kidney-streptococcus mixtures. The interval between series of injections was two to three months.

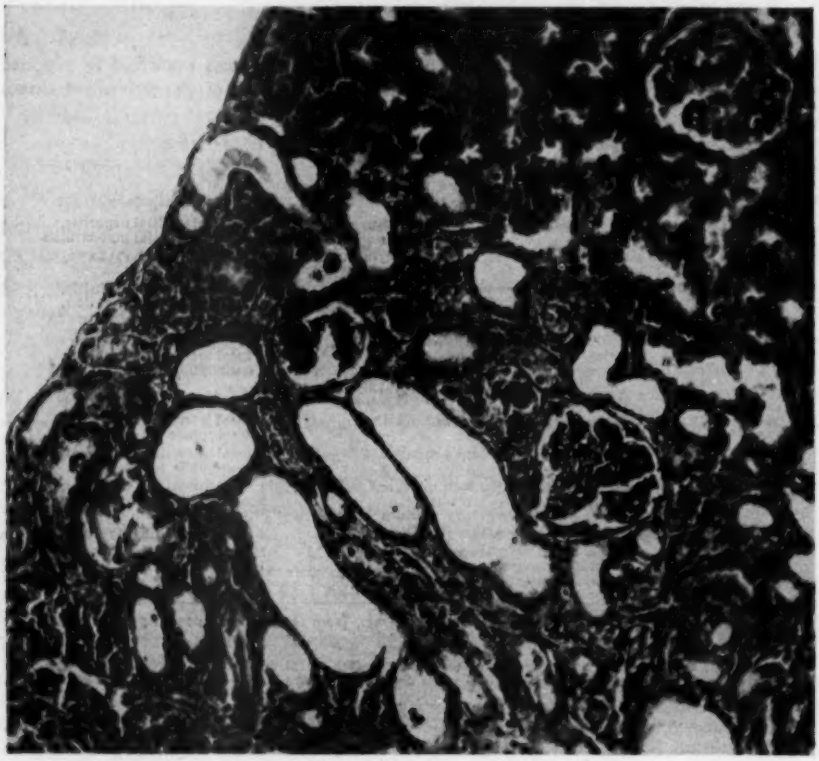

Fig. 1.—Focal interstitial nephritis from rat No. 165, Group 1A, showing a mild chronic inflammatory reaction involving two glomeruli, with cystic dilatation of many tubules. Killed at 385 days. Hematoxylin and eosin; × 400.

Crystalline desoxycorticosterone acetate [15] was dissolved in acetone and mixed with peanut oil, and the acetone was removed by distillation so that the final concentration of desoxycorticosterone acetate was 10 mg. per milliliter in peanut oil. Injections of 2.5 mg. were given subcutaneously daily into the skin of the back.

Base-line urine analyses were made, and specimens of urine were collected at three to four week intervals and tested for albumin by the method of Shevky and Stafford.[16] The urinary sedi-

15. Desoxycorticosterone acetate was supplied by Dr. Kenneth Thompson, of Roche-Organon, Nutley, N. J.

16. Shevky, M. C., and Stafford, D. D.: Laboratory Methods of the United States Army, Ed 4, Philadelphia, Lea & Febiger, 1935.

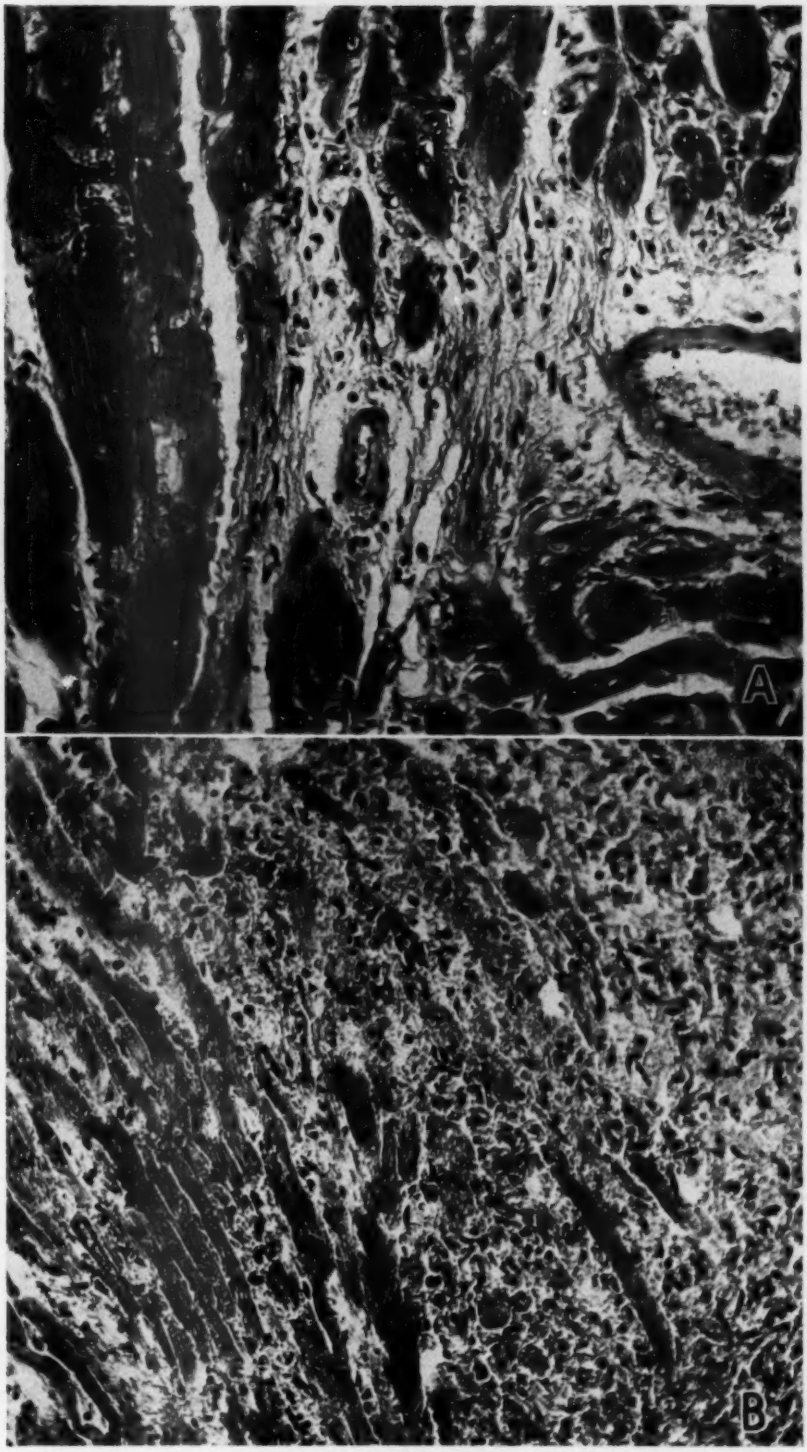

Fig. 2.—Focal interstitial myocarditis. *A*, rat No. 237, control Group IV, showing minimal myofibrillar degeneration and a mild inflammatory reaction. Killed at 385 days. Hematoxylin and eosin; × 400. *B*, rat No. 329, Group IIIC, showing a portion of an area of advanced myocardial fibrosis. This animal was found dead at 116 days. Hematoxylin and eosin; × 400.

ment was examined microscopically whenever the degree of albuminuria exceeded normal limits (5 gm. per liter). After 8 to 12 months of observation, the animals were killed with ether and examined (except one rat which died at 116 days); sections of organs were taken for microscopic study, and serum was obtained for urea nitrogen determinations. Capillary tube precipitin tests were also run on aliquots of the sera, with saline organ extracts in concentrations varying from 1:5 to 1:160, according to the technique of Lancefield and Swift.[17]

RESULTS

Reference to the Table, Group I, will show that 40 of the 103 experimental animals were given injections of homologous kidney in Freund adjuvant. Only

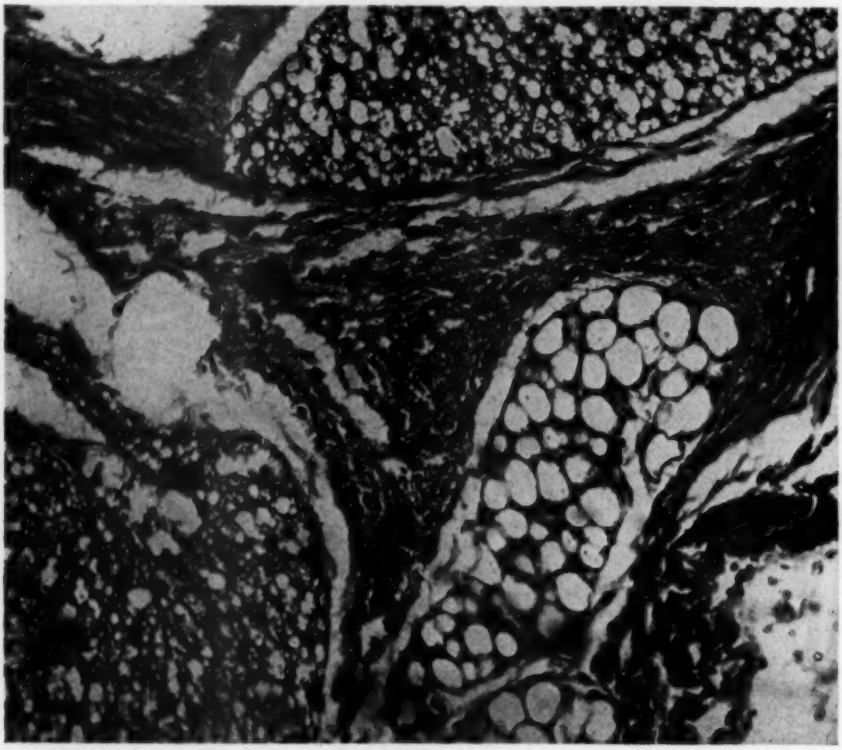

Fig. 3.—Eosinophilic inflammation in the periadrenal adipose tissue of rat No. 225, Group IC. The outstanding features are edematous eosinophilic collagen with interspersed precipitated fibrin and a predominantly eosinophilic cellular infiltration. Killed at 326 days. Hematoxylin and eosin; × 400.

two of these animals had significant proteinuria, which was accompanied on two occasions by cylindruria. When the animals were exsanguinated at the conclusion of the experiment, no nitrogen retention was found. At autopsy no gross or microscopic lesions characteristic of glomerulonephritis were noted in either of these 2 animals or in any other of the 40 rats. The proteinuria could be accounted for

17. Lancefield, R. C., and Swift, H. F., in Bacterial and Mycotic Infections of Man, edited by R. J. Dubos, Philadelphia, J. B. Lippincott Company, 1948.

in one of the animals on the basis of existing pyelonephritis. In the other animal a few casts were found in the tubules, but no other pathological changes could be recognized microscopically.

Twenty-eight rats, Group III, which were given injections of kidney-streptococcus mixtures also exhibited no evidence of glomerulonephritis, either during life or at the time of autopsy.

It may be seen by reference to the Table that 35 control animals, Groups II and IV, were given injections either with heart or placenta in Freund adjuvant,

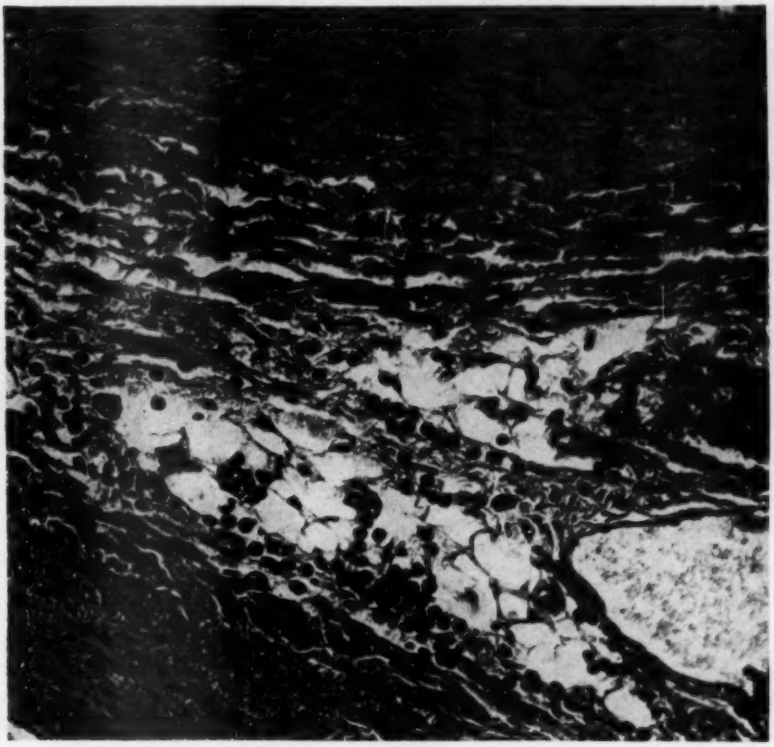

Fig. 4.—Eosinophilic inflammation in connective tissue at the base of the heart of rat No. 247, control Group IID. The lesion is located between two great vessels and has the same characteristics as described in Figure 3. Killed at 283 days. Hematoxylin and eosin; × 400.

with the adjuvant alone, with desoxycorticosterone acetate alone, or with three courses of ether-killed hemolytic streptococci. In none of these controls did clinical or histopathologic evidence of glomerulonephritis develop.

A variety of lesions apparently unrelated to the experimental procedures were observed in all four groups of rats, as may be seen by reference to the Table. Multiple lesions of focal interstitial nephritis (Fig. 1) seen in 11 rats consisted of small areas of mononuclear cell infiltration, usually associated with fibrosis and hyalinization of glomeruli and dilatation of tubules in the involved area. Focal

interstitial myocarditis (Fig. 2*A*), noted in seven animals, was characterized by small areas of myofibrillar degeneration, infiltration with mononuclear cells, and occasional collections of polymorphonuclear leucocytes and fibroblasts. In three rats, more advanced areas of dense fibrosis were scattered through the myocardium (Fig. 2*B*). It is noteworthy that no cardiac lesions occurred in the control group, Group IIA, receiving heart-adjuvant mixture.

Lesions characterized by edematous eosinophilic collagen with interspersed precipitated fibrin and numerous eosinophiles, mononuclear cells, occasional poly-

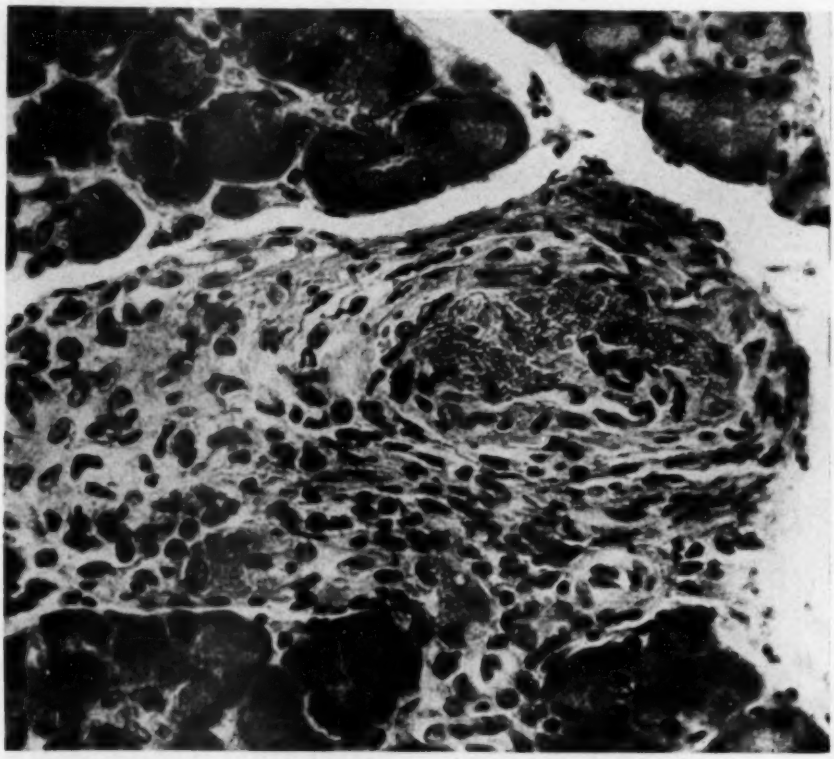

Fig. 5.—Periarteritis involving a medium-sized pancreatic artery of rat No. 235, control Group IV. Killed at 387 days. Hematoxylin and eosin; × 400.

morphonuclears, fibroblasts, and proliferating capillaries were found in the mesentery and in the pericardial, perirenal, and periadrenal adipose tissue of six animals (Figs. 3 and 4).

As recorded in the Table, an isolated instance of periarteritis with the usual pathological features was found in a medium-sized pancreatic artery in one rat. (Fig. 5).

The adrenal glands of all animals appeared normal, and no evidence of subcapsular atrophy was found in the desoxycorticosterone-acetate-treated rats at the time of death, one to four months after the injections had been discontinued.

Precipitins to saline extracts of rat kidney, heart, or placenta were not found in the serum of any of the animals by the qualitative technique employed.

COMMENT

The injection of homologous kidney admixed with Freund adjuvants failed to produce glomerulonephritis in the rat. This is in sharp contrast to the relative ease with which experimental disseminated encephalomyelitis may be produced in monkeys, dogs, guinea pigs, mice, and rats [4] by immunization with brain and adjuvants and clearly indicates the need for more detailed study of the nature of the response in various animal species to injected homologous kidney.

No evidence of glomerulonephritis was noted in rats given injections of killed streptococci and homologous kidney according to the technique of Cavelti, although the experimental procedure presumably should have caused the maximal development of lesions. Underlying this discrepancy of results there must be several factors of fundamental importance. For example, although Cavelti employed animals of the Long-Evans strain, it is conceivable that inbreeding may have yielded a strain of animals endowed with genetic susceptibility to glomerulonephritis, evidence of which could be evoked following appropriate stimulation. The work of Kirschbaum and others [18] on an inbred strain of mice, a large proportion of which developed spontaneous glomerulonephritis, lends some credence to this speculation. The strain difference in susceptibility to nephrotoxic nephritis in the rat, reported by Smadel and others,[19] also supports this possibility. Additional variables are the strains of streptococci used, their relative virulence, and possible minor differences in cultural methods employed. For example, Cavelti found that rabbits given injections intraperitoneally with the killed organisms died,[20] a finding that could not be duplicated with the organisms used in the present experiment. Toward the end of the growth period on Bernheimer's medium, the same author did not neutralize the acids formed, and in collecting the organisms some toxic products at or near their isoelectric point may have been precipitated and included in the centrifuged sediment. Another variable is the effect of storage temperature on the antigenicity of kidney extracts, since Sprunt and Dulaney [21] found that increased isoantigenicity roughly correlated with the degree of autolysis of rabbit kidney extracts stored at 4 C. The absence of the capacity to stimulate isoantibody formation by extracts stored at —20 C. further indicates the possible importance of autolytic changes in rendering the tissue antigenic.

None of the various pathological changes noted can be related with certainty to the experimental procedure. Focal interstitial nephritis was found in 11 rats in the present study. Such focal lesions probably represent healed areas of pyelonephritis, and this lesion is uncommon in rats dying before 700 days of age.[22]

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Seven rats in this series had myocardial damage. In a study of a large number of rats of the Osborn-Mendel stock, Wilens and Sproul [22] found "spontaneous" cardiovascular disease. In their series, myocardial fibrosis, with or without interstitial myocarditis, occurred in 59.9% of the rats. Significant degrees of myocardial fibrosis, however, were not seen in animals less than 600 days old at death. None of the seven cases of myocarditis in the present investigations occurred in the eight rats given injections of the heart adjuvant mixture. The finding in this small series of animals is contrary to the observations of Frick,[5] who described lesions of interstitial myocarditis in rats treated with homologous heart tissue in adjuvants.

It is evident from the observations reported here that certain nonspecific pathological changes were found in the animals of these experiments, but the presence of the lesions seemed premature at ages ranging up to 400 days when compared to untreated animals. The impairment of general health and the debility which may be associated with an experimental regimen may, however, initiate the earlier appearance of lesions which commonly become manifest in later life.

SUMMARY

Glomerulonephritis did not develop in rats given injections intramuscularly or subcutaneously with homologous renal tissue in Freund adjuvants. Control animals given injections of homologous heart-adjuvant and placenta-adjuvant mixtures failed to acquire cardiac or renal lesions. Lesions of glomerulonephritis did not develop in rats given injections intraperitoneally with mixtures of homologous renal tissue and Group A hemolytic streptococci. Simultaneous administration of desoxycorticosterone acetate and NaCl to animals receiving kidney-adjuvant or kidney-streptococcus mixtures did not influence the results. The use of different serological types of Group A streptococci in successive injections with homologous renal tissue did not evoke the lesions of glomerulonephritis. Precipitins to homologous renal, cardiac, and placental tissue were not demonstrable by the qualitative technique employed. A variety of histopathological lesions was noted. These lesions did not have a discernible relationship to the experimental procedure used. Though similar to lesions reported in older rats, they appeared at an earlier age. Several possible reasons for the differences in results obtained by Cavelti and those of the present experiment are discussed.

POTASSIUM DICHROMATE POISONING AND REPEATED POISONING WITH URANYL NITRATE

Effect on Serum Lipids of Rabbits

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HYPERLIPEMIA in patients with the nephrotic syndrome has long been recognized, but the significance of the kidneys in the pathogenesis of this condition is not clear. However, in animals damage to the kidneys produced experimentally in a variety of ways is followed by transient hyperlipemia. The term "hyperlipemia" is used here to mean an abnormal increase of the neutral fat in the serum, in contrast with the terms "hyperlecithinemia" and "hypercholesterolemia" which are used to designate an increase of these respective lipids in the serum.[1]

Rabbits poisoned with uranium have retrogressive changes and even necrosis of the epithelial cells lining the convoluted tubules of the kidney. The accompanying fatty changes of the liver are considered due to acidosis and azotemia.[2] This is associated with a transient hyperlipemia, the neutral fat fraction being the most elevated.[3] Dogs so poisoned also have an increase in the total blood lipid and cholesterol.[4] Heymann and Clark [4] found that subcutaneous or intramuscular

This investigation was aided by the Otho S. A. Sprague Memorial Fund.

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Dr. Johnson was the John Jay Borland Fellow, St. Luke's Hospital, and is now with the Department of Pathology, University of Illinois.

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injection of mercury bichloride in dogs is followed by retrogressive changes of the epithelium lining the tubules of the kidneys and a transient elevation of both total lipid and cholesterol. Degenerative changes of the tubular epithelium of the kidneys occur also with potassium dichromate poisoning, and dogs so poisoned have a transient hyperlipemia with an increase of total lipid and cholesterol.[4] Acute tubular damage produced by irradiation of a surgically exposed kidney is followed by fibrous tissue replacement of the destroyed tubules and some tubular regeneration. Glomerular changes are minimal.[5] Blood cholesterol is elevated during the acute stage and falls to approximately normal limits when the lesions become chronic. Glomerular and degenerative changes of the cells lining the renal tubules occur in rats following the injection of purified nephrotoxic gamma globulin prepared from rabbit anti-rat-kidney serum. Four weeks after the injection, serum cholesterol and total serum lipid concentration, as well as the weight of the adrenal glands and liver, increased in parallel with the severity of the nephritis.[6] Experimental unilateral occlusion of the renal blood vessels in rabbits is followed by transient hyperlipemia, although the opposite kidney is not injured. The phospholipid and cholesterol fractions are elevated, but the greatest increase is in the neutral fat fraction. When ischemia is prolonged, the hyperlipemia may be marked and the renal necrosis extensive.[7]

The sensitivity of the tubular epithelium to uranium is decreased by previous renal damage. Repeated doses of uranium, according to Hunter,[8] may be given without injury, although damage of the kidneys occurred with the initial dose of uranium. In these so-called "immunized kidneys" of rabbits he found little of the original epithelium in the susceptible part of the tubules. It had been replaced by cells which had no histological evidence of injury with repeated injections of uranium. In dogs, MacNider[9] found that uranium produced degeneration of epithelium of the proximal convoluted tubules. Repair of this epithelium was accomplished both by regeneration of mildly injured tubule cells and by an ingrowth of cells from the terminal portion of the proximal convoluted tubule or from Henle's loops. This regenerated epithelium was low and flattened, as compared with the normal epithelium, and was resistant to another dose of uranium. MacNider considered this a less specialized form of epithelium. Uranium nitrate given to rabbits with unilateral hydronephrosis following ligation of one ureter caused extensive necrosis of the epithelium lining the proximal convoluted tubules in the normal kidney, but the hydronephrotic kidney had only minimal changes.[10]

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If degeneration of renal tubule epithelium is a factor in the production of the lipemia with uranium poisoning, a second dose of uranium might produce less lipemia, since the regenerated epithelium is resistant to uranium. The purpose of this study was to find if repeated injections of uranium produce as much lipemia as the initial injection and also to study the effects of potassium dichromate poisoning on the serum lipids in rabbits.

MATERIAL AND METHODS

Rabbit serum lipids and blood nonprotein nitrogen were determined preceding and at one- to three-day intervals following the intravenous injection of potassium dichromate and uranyl nitrate. Rabbits were bled from the marginal ear vein, and chemical analyses of the blood were performed according to the methods described.[3] Any effect on the blood lipids by the loss of blood through bleeding was controlled by bleeding another animal simultaneously. White male rabbits weighing between 6 and 9 lb. (2.7 and 4.1 kg.) were used.

Changes in Serum Esterified-Fatty-Acid Concentration Following Injection of Potassium Dichromate

Rabbit	Control Serum Esterified-Fatty-Acid Concentration, mEq./L.	Mg. of $K_2Cr_2O_7$ Injected Intravenously	Serum Esterified-Fatty-Acid Concentration 2 to 7 Days After $K_2Cr_2O_7$ Injection, mEq./L.
B 4-6	4.2	40	28.7
B 5-3	6.4	30	21.2
B 5-4	6.7	30	17.3
UN 11	8.3	35	22.1
E 3	4.9	35	13.0
E 19	4.7	35	14.3
E 2	4.9	35	11.3
E 23	3.5	35	7.8
E 31	3.8	35	8.7
E 33	5.9	35	20.9
E 35	6.3	35	47.8
E 36	4.0	35	39.8

Potassium Dichromate Poisoning.—Potassium dichromate solution containing 10 mg. per milliliter was injected intravenously in amounts between 10 and 60 mg. In 10 of 12 rabbits given 30 to 40 mg. of potassium dichromate intravenously, the serum esterified fatty acids increased to more than twice the control level during the following week (Table). In two rabbits (E23 and E31) the serum lipid changes were not considered significant. The serum esterified fatty acids began to rise one to three days after the potassium dichromate injection and reached a peak in two and seven days. In those that survived, serum fatty acids returned toward the control level in one to three weeks. Three animals (E3, E33, and E36) survived less than one week after the potassium dichromate injection. In two animals that received only 10 mg. of potassium dichromate there was no appreciable increase of the fatty acids. When these animals later were given 30 mg. of potassium dichromate, there was a small elevation of fatty acids, appreciably less than the elevation occurring in the rabbits given an initial injection of 30 mg. In Chart 1 are plotted changes of the various lipid fractions in a typical experiment (rabbit UN 11) following the intravenous injection of 35 mg. of potassium dichromate. The maximum increase was in the fatty acid fraction attributed mainly to the neutral fat, although the other lipid fractions participated in the increase. The blood nonprotein nitrogen increased and decreased in parallel with the serum lipid changes.

Rabbit B4-6 died seven days after the injection of 40 mg. of potassium dichromate and one day after the serum esterified fatty acid had reached a level of 28.7 mEq. per liter. Only the

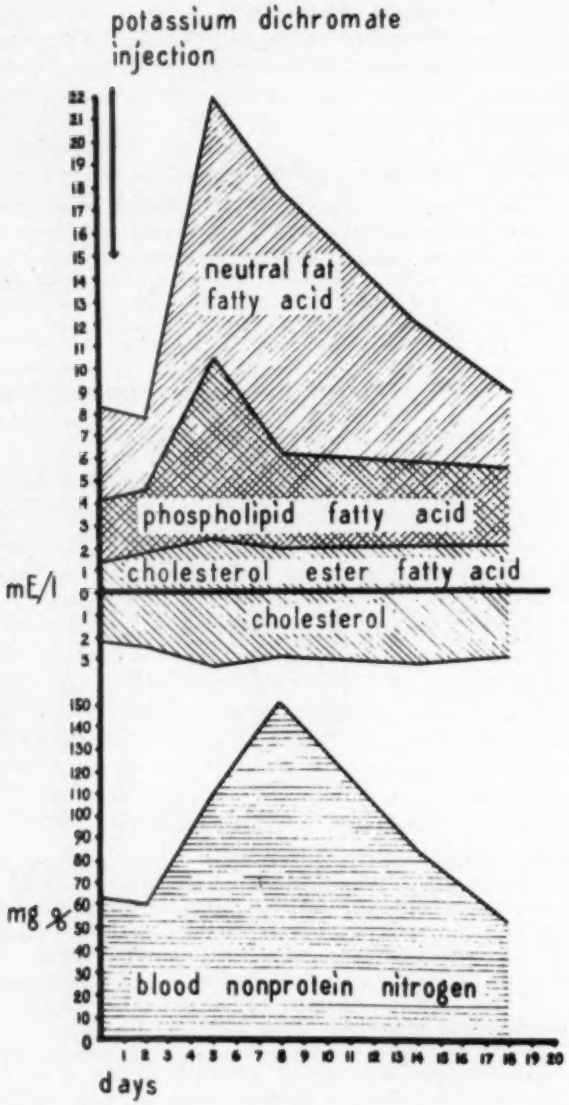

Chart 1.—Changes in serum lipid fractions in rabbit UN 11 following intravenous injection of 35 mg. of potassium dichromate.

basement membranes remained in many of the convoluted tubules of the kidneys of the animal, and the lumens contained granular acidophilic material. In some tubules a few lining cells with pyknotic nuclei remained, and in others the lining epithelium was intact. Glomeruli were well preserved, although there was some increase in cellularity. The two rabbits (E23

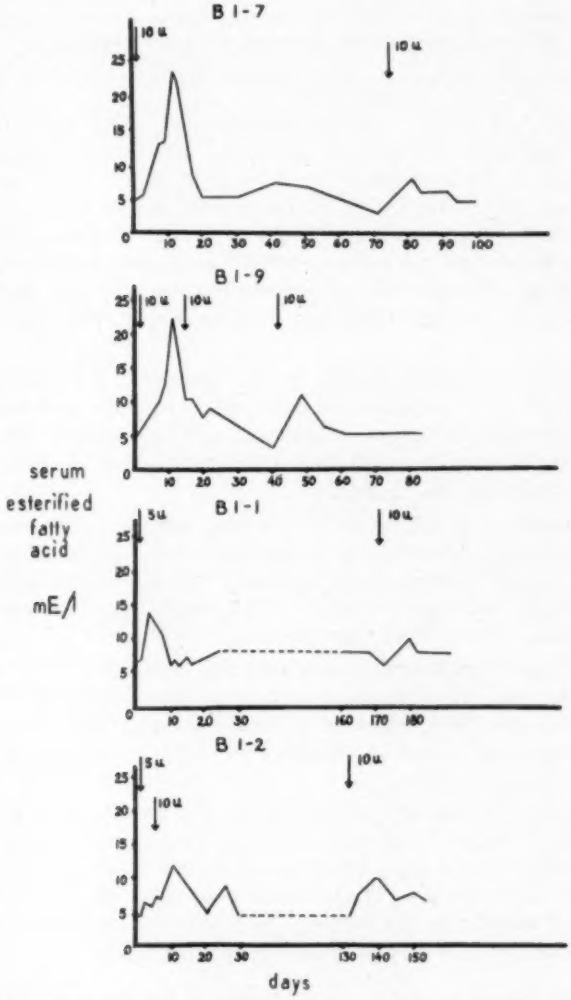

Chart 2.—Changes in serum esterified fatty acids in four rabbits following repeated injections of 5 and 10 mg. of uranyl nitrate as indicated.

and E31) that did not have appreciable lipemia or elevation of the blood nonprotein nitrogen following the injection of potassium dichromate were killed by air embolism 19 days (E31) and 7 weeks (E23) later. The cells lining the convoluted tubules and the other structures of the kidneys were without appreciable changes.

Repeated Uranyl Nitrate Poisoning.—Bauer, Johnson, Carbonaro, and Hirsch found that a single subcutaneous or intravenous injection of 10 mg. of uranyl nitrate produced a transient rise of the serum esterified fatty acid in rabbits. In Chart 2 are plotted the serum esterified fatty acid levels of four rabbits with repeated injections of uranyl nitrate. Although an initial injection of 10 mg. of the uranium produced a significant rise in the serum esterified fatty acids, a second and third injection of 10 mg. of uranyl nitrate produced only minimal elevations of these fatty acids. If the initial injection was only 5 mg. of uranium, a second and third injection of 10 mg. also produced minimal elevation of the serum fatty acids.

COMMENT

Bing, Heckscher and Jessen [11] injected subcutaneously total doses of 0.449, 0.352, 0.617, and 0.628 gm. of potassium dichromate into rabbits. These amounts were divided into 33 to 58 injections given in 55 to 78 days. In one animal the blood "fat cholesterol" rose from 0.04 to 0.23%, and in another it rose from 0.09 to 0.23%. No significant changes occurred in the other two. Heymann and Clark [4] injected potassium dichromate into a dog and found an increase in the serum total fat from a level between 420 and 580 to 1,600 mg. per 100 ml. before death about 5 days later. Total serum cholesterol increased from between 40 and 110 to 216 mg. per 100 ml.

According to Oliver, MacDowell, and Tracy,[12] damage to the proximal convoluted tubules resulting from uranium and potassium dichromate poisoning occurs evenly in every nephron of the kidneys. In addition, with large doses of the nephrotoxic metals, Oliver found random focal disruptive tubular lesions of the type associated with focal cortical ischemia of shock.

Transient hyperlipemia in rabbits occurs following damage to the renal tubule epithelium produced experimentally by potassium dichromate, uranyl nitrate,[3] mercury bichloride,[4] roentgen irradiation of a surgically exposed kidney,[5] nephrotoxic anti-rat-kidney serum,[6] and renal ischemia.[7] When neutral fat is estimated, it is regularly the fraction of serum lipids most elevated.

The absence of significant lipemia with second and third injections of uranium in rabbits correlates with an increased resistance of renal tubule epithelium to repeated injections of uranium, as observed by Hunter and MacNider. Accordingly, this suggests that the hyperlipemia of uranium poisoning is consequent to damage of the cells lining the renal tubules.

The hyperlipemia following heavy metal poisoning is considered to be part of a systemic disturbance of lipid metabolism resulting from a disorder of some factor controlling mobilization of the triglycerides of depot fat.[3] The lipemia following renal ischemia and other forms of renal damage may be of similar significance. Dible and Popjak [13] found that fatty changes in kidneys of rabbits produced by starvation result from an infiltration of lipids from the fat depots.

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SUMMARY

Intravenous injection of 30 mg. or more of potassium dichromate produced a transient hyperlipemia in 10 out of 12 rabbits. The greatest increase was in the esterified fatty acid fraction of the serum lipid and is attributed to neutral fat. Cholesterol, cholesterol ester, and phospholipid were increased but only in small amounts.

Second and third injections of uranyl nitrate produced significantly less elevation of serum lipids than the similar initial dose of uranium. In view of the increased resistance of renal tubule epithelium to repeated injections of uranium, this suggests that hyperlipemia of uranium poisoning is consequent to damage of the cells lining the renal tubules.

Case Reports

ANOMALOUS ORIGIN OF LEFT CORONARY ARTERY FROM PULMONARY ARTERY (BLAND-WHITE-GARLAND SYNDROME)

Report of Two Cases

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THE ANOMALOUS origin of the left coronary from the pulmonary artery is not a common developmental defect of the heart. In a recent review of the literature Wüthrich [1] found 50 cases, and he reported 2 of his own. His report included cases of infants and adults with and without other congenital defects of the heart.

The report is limited to infantile cases in which the origin of the left coronary from the pulmonary is the only developmental defect of the heart. In this series there are approximately 32 cases reported in the literature.[2]

From the Department of Pathology, St. Bernard's Hospital, Chicago.

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(Footnote continued on next page)

The unusual vascular anomaly present is a natural experiment which allows the study of myocardial changes resulting from low-pressure inadequately oxygenated blood.

This report adds two more cases to the series:

REPORT OF CASES

CASE 1.—*History.*—A 3-month-old girl was admitted to the hospital because of grunting respirations, limpness, and cyanosis of 12 hours' duration. The birth and immediate postnatal condition of the child were regarded as normal. The baby was breast-fed for two months, but during that time supplemental feedings were given because of a slow gain in weight. Two weeks later a pediatrician was consulted, and the parents were told that the infant had congenital heart disease, because a cardiac murmur was detected on physical examination. Three days before admission into the hospital, the child became irritable, cried when handled, and became blue about the mouth and fingers. The infant nursed well but always vomited parts of the feeding. Twelve hours before hospitalization, her respirations became more rapid and grunting and cyanosis increased. Physical examination at this time revealed a female infant in a moribund condition, with cyanosis, shallow and grunting respirations, and a heart rate of about 180 per minute. Besides being rapid, the heart sounds were forceful. Moist râles were heard over the lower two-thirds of the right chest posteriorly. A diagnosis of pneumonitis and congenital heart disease was made. The infant died 30 minutes after admission.

Postmortem Examination.—Autopsy was performed two hours after death. The essential gross anatomic abnormalities were as follows: anomalous origin of the left coronary artery from the pulmonary artery; marked hypertrophy of the myocardium of both ventricles of the heart; extensive subendocardial fibrosis of the myocardium of the left ventricle of the heart; moderate bilateral hydrothorax; small subpleural hemorrhages of both lungs; focal atelectasis of both lungs.

The heart in its position in the thorax was markedly enlarged. It had a maximum transverse diameter of 9.0 cm. and an oblique diameter of 6.0 cm. from the right auricle above to the apex of the left ventricle below. It weighed 75 gm. (Fig. 1). The pericardium was smooth, and the epicardium contained a small amount of fat. The circumference of the pulmonic ring was 3.0 cm. The leaflets of the pulmonic valve were thin. The left coronary artery arose from the pulmonary artery slightly above the left pulmonic cusp. The thickness of the myocardium of the right ventricle at the conus was 4.0 mm. The circumference of the tricuspid ring was 5.0 cm. The tricuspid valve leaflets were thin. The lining of the right ventricle, auricle, and auricular appendage was smooth, and these chambers were dilated. The myocardium of the right ventricle was uniformly red-brown. The lining of the root of the aorta was smooth. The circumference of the aortic ring was 2.0 cm.; of the mitral, 3.5 cm. The orifice of the right

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linken Kranzarterie aus der Lungenschlagader, Frankfurt. Ztschr. Path. **57**:265, 1942. (*r*) Soloff, L. A.: Anomalous Coronary Arteries Arising from Pulmonary Artery, Am. Heart J. **24**:118, 1942. (*s*) Proescher, F., and Baumann, F. W.: Abnormal Origin of Left Coronary Artery with Extensive Cardiac Changes, J. Pediat. **25**:344, 1944. (*t*) Tichenor, C. J.: Sudden Death from Coronary Insufficiency: Report of a Case in an Infant, Clin. Proc. Child. Hosp. **2**:92, 1946. (*u*) Eidlow, S., and Mackenzie, E. R.: Anomalous Origin of Left Coronary Artery from Pulmonary Artery: Report of a Case Diagnosed Clinically and Confirmed by Necropsy, Am. Heart J. **32**:243, 1946. (*v*) Lyon, R. A.; Johansmann, R. J., and Dodd, K.: Anomalous Origin of Left Coronary Artery, Am. J. Dis. Child. **72**:675, 1946. (*w*) Kaumitz, P. E.: Origin of Left Coronary Artery from Pulmonary Artery: Review of Literature and Report of 2 Cases, Am. Heart J. **33**:182, 1947. (*x*) Gasul, B. M., and Loeffler, E.: Anomalous Origin of the Left Coronary Artery from the Pulmonary Artery (Bland-White-Garland Syndrome): Report of 4 Cases, Pediatrics **4**:498, 1949. (*y*) Fishee, H., and Lloyd, O. C.: A Case of Anomalous Left Coronary Artery from Pulmonary Artery, Brit. Heart J. **13**:406, 1951. (*z*) de Chastonay, E., and Buser, M.: Über einen Fall von Abgang der Arteria coronaria sinistra aus der Arteria pulmonalis, Helvet. paediat. acta **4**:308, 1949.

Fig. 1 (Case 1).—Opened heart, showing the origin of the left coronary artery from the pulmonary artery. The left coronary artery orifice can be seen above the left pulmonic cusp. The artery is continuous to the apex. The wall of the left ventricle is hypertrophied, and the lighter subendocardial region represents fibrosis.

Fig. 2 (Case 1).—Section from the lining of the left ventricle. The endocardium is thickened with fibrosis tissue. The muscle bundles of the myocardium are replaced at many levels by fibrosis tissue with deposits of calcium. The remaining muscle fibers are hypertrophied. Van Gieson-Weigert stain; × 70.

coronary artery arose in the aorta and was in its usual position. The lining of the left ventricle and auricle was thickened with gray-white tissue and the chambers moderately dilated. The papillary muscles of the left ventricle were hypertrophied, mottled with gray scars. The chordae tendineae were thin. The myocardium of the anterior wall near the septum and extending upward from the apex to the midportion of the left ventricle was pale brown with regions of gray-white scars visible only near the endocardium. The myocardium of the posterior wall of the left ventricle was red-brown and not visibly scarred. The coronary artery arising from the pulmonary artery divided soon after its origin to form the descending and circumflex branches. The descending branch continued downward over the septum to the apex. The circumflex branch was small and followed its usual course. The right coronary artery had its usual distribution. The lumens of the coronary arteries were patent and the linings smooth. A roentgenogram of the heart after removal disclosed thin linear opaque streaks in the papillary muscles. Here calcific deposits were found microscopically.

Histologic Findings.—Many sections of the heart stained with hematoxylin and eosin and by the Weigert elastic tissue and Van Gieson methods were examined.

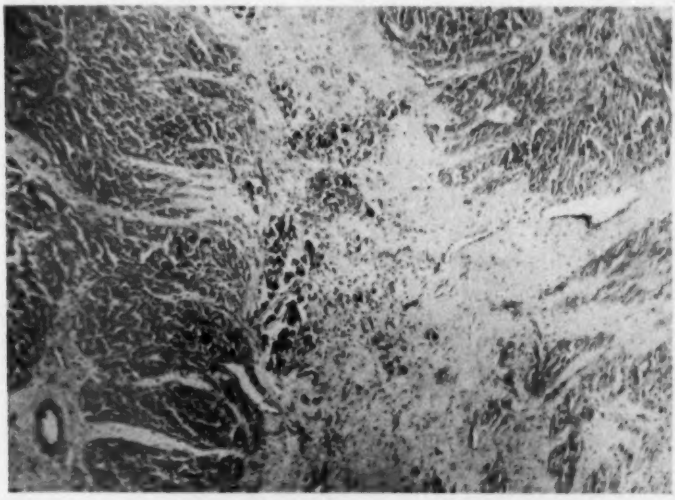

Fig. 3 (Case 1).—Section of the myocardium of the wall of the left ventricle. There is a considerable replacement of the myocardial muscle bundles of the heart with calcific deposits. Hematoxylin and eosin stain; × 70.

These tissues were made from blocks cut at various levels from both ventricles, ventricular septum, and auricles. In sections from the anterior wall of the left ventricle near the apex and septum there was an extensive replacement of the myocardial cells by fibrous tissue. This was greatest near the endocardium. The scarring was absent in the tissues adjacent to the epicardium. Scarring was greatest in the papillary muscles (Fig. 2). In the regions where the fibrous tissue replacement of the myocardium was greatest there were many focal deposits of calcium (Fig. 3). In the heart muscle of the lateral wall of the left ventricle similar changes were present but to a less degree. There were neither fibrous nor calcareous changes seen in the myocardium of the posterior wall of the left ventricle, the right ventricle, or the left and right auricles. The intraventricular septum had a moderate fibrous tissue

replacement near the subendocardial region toward the left ventricular side. Sections of all the coronary arteries had no noteworthy changes but did have the typical structure of an artery.

CASE 2.—*History.*—A 2-month-old boy died unexpectedly at home, and the case was referred to the coroner because no physician had been in attendance.

Postmortem Examination.—The postmortem examination revealed that the essential changes were confined to the heart. It was large in its position in the thorax. The pericardium was smooth; the epicardium contained a moderate amount of fat. The heart weighed 40 gm. The lining of the pulmonary artery was smooth. The mouth of the left coronary artery was present in the pulmonary artery just above the level of one of the semilunar pulmonic cusps. It was 2.0 mm. in diameter. The thickness of the myocardium of the right ventricle near the pulmonic ring was 2.0 mm. The chambers of the right side of the heart were dilated. The lining of the right auricle and ventricle and that of the aorta were smooth. The mouth of the right coronary artery had its usual position in the wall of the aorta. The aortic and mitral leaflets were thin. The left auricle and ventricle were dilated. The myocardium of the left ventricle at the level of the mitral ring was 0.8 cm. thick. The myocardium was red-brown and mottled with several small gray-white regions of fibrosis. These were confined to the wall of the left ventricle near the endocardium and anteriorly. The myocardium of the right ventricle was unchanged. The lining of the left ventricle was thickened with fibrous tissue. The left coronary artery divided soon after its origin into the left descending and circumflex branches. These arteries after division followed their usual course. The right coronary artery had a normal distribution. The descending branch of the left coronary artery was more prominent than the other coronary arteries.

Histology.—The changes of the heart muscle were confined to the left ventricle, especially the anterior and lateral portions. Here there were many regions of fibrous tissue replacement of the myocardial cells similar in distribution to those described in the previous case. These changes were greatest near the endocardium and diminished toward the epicardium. There were no regions of calcification in the fibrous tissue. There were no unusual changes of the myocardial fibers of the right ventricle, posterior wall of the left ventricle, auricles, or coronary arteries.

COMMENT

In 1911 Abrisikoff [2b] reported the first case of this anomaly. In 1933 Bland, White and Garland [2j] reported a case and found eight others in the literature. They were the first to suggest the possibility of a clinical diagnosis.

In 1947 Kaunitz [2w] reviewed the literature. In all cases reported there were similar clinical and pathological findings. Clinically the patients are usually born at full term and their deliveries are normal. They develop normally until the age of 2 to 3 months, when dyspnea, wheezing and cyanosis associated commonly with the exertion of feedings gradually begin to appear. They have tachycardia. Electrocardiograms show inversion of T waves, low amplitude of QRS complexes, and no axis deviation. The cases terminate usually with pulmonary complications or heart failure. In recent years a clinical diagnosis has been made by Eidlow, Mackenzie [2u] and Gasul and Loeffler.[2x] Reports of the disorder, found unexpectedly at autopsy in seven adults, has been reviewed by Kaunitz.[2w] Gouley [3] recently reported a case in an adult.

3. Gouley, B. A.: Anomalous Left Coronary Artery Arising from Pulmonary Artery (Adult Type), Am. Heart J. **40**:630, 1950.

The pathological findings in all infant cases are smiliar. Myocardial hypertrophy and subendocardial fibrosis are present. Aneurysmal dilatation and mural thrombi of the left ventricle are not uncommonly found. Myocardial changes range from acute necrosis to dense scarring and calcification.

The question arises as to the mechanism of the cardiac hypertrophy and fibrosis. These changes are not considered to start in utero, since there is little or no difference in the oxygen content of the blood in the aorta and the pulmonary artery before birth. The etiological factors present are the inadequately oxygenated venous blood and the low pressure from the pulmonary artery. Levine and Ford [4] reported six cases in adults with endocardial infarcts of the heart caused by global restriction of the coronary blood flow. They suggest that the global restriction was caused by other factors besides vessel obstruction, such as anemia, shock, ostial disease, and atherosclerosis without occlusion. In their cases electrocardiograms showed T wave and RS-T segment changes. The infarcts occurred in the subendocardial zone, where the blood supply was the poorest. The degenerative changes in the hearts of the infants were also greatest in the endocardial region, the "global restriction" in the infant cases being the low pressure venous blood supply. A possible sequence of events is as follows: With inadequately oxygenated blood supply, the left ventricle has a temporary diminution in contractile power, due perhaps to toxic metabolic factors. The weakened ventricle is unable to empty completely; the residue remaining increases the size of the heart in diastole and thus the rigor of contraction.

The continuance of augmented contractions leads to hypertrophy. Degeneration and subsequent fibrosis follow owing to the progressively increased oxygen requirement. Thus a vicious circle develops and ends when the limit of the cardiac reserve is reached. Gasul and Loeffler [2x] suggest that dilatation of the left ventricle causes compression of the coronary artery branches, and this plays an important role in producing the myocardial fibrosis. In a recent report, Johnson [5] suggests that anoxia resulting from cardiac malformations may have been an important factor in the production of endocardial fibroelastosis in infant hearts.

SUMMARY

Two instances of anomalous origin of the left coronary artery from the pulmonary artery in infants 2 and 3 months of age are reported.

The possible pathogenesis of the accompanying left ventricular hypertrophy and fibrosis is discussed.

4. Levine, H. D., and Ford, R. V.: Subendocardial Infarction: Report of 6 Cases and Critical Survey of the Literature, Circulation **1**:246, 1950.

5. Johnson, F. R.: Anoxia as a Cause of Endocardial Fibroelastosis in Infancy, A. M. A. Arch. Path. **54**:237, 1952.

General Reviews

CULTIVATION OF VIRUSES

A Critical Review

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AND
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Group I: Viruses which have propagated in tissue culture (all criteria have been met).

Coxsackie
Encephalitides
  Japanese B
  St. Louis
  West Nile
  Semliki Forest fever
  Russian spring-summer
  Eastern equine encephalomyelitis
  Western equine encephalomyelitis
  Venezuelan equine encephalomyelitis
Foot and mouth
Fowl leucosis
Fowl plague
Herpes simplex
Hog cholera
Influenza
Lymphogranuloma venereum
Lymphocytic choriomeningitis
Measles
Mouse encephalomyocarditis
  Col-SK
  MM
Mouse encephalomyelitis (Theiler's GD VII virus)
Mumps
Newcastle disease
Poliomyelitis
Pseudorabies
Psittacosis
Rabbit myxoma
Rabies
Rous sarcoma
Rift Valley fever
Vaccinia
Variola
Vesicular stomatitis
Virus III
Yellow fever

Group II: Viruses which have doubtfully or temporarily propagated in tissue culture (one or more criteria have not been met)

"Anopheles A and B"
Avian encephalomyelitis
Common cold
Ectromelia
Ilhéos
Epidemic keratoconjunctivitis
Rabbit fibroma (inflammatory strain)
Fowl pox
Infectious hepatitis
Louping ill
Sheep pox
Varicella

Group III: Viruses which have not propagated in tissue culture

Herpes zoster
Inclusion blennorrhea
Infectious mononucleosis
Rabbit fibroma (fibromatous strain)
Sandfly fever
Teschen's disease
Trachoma

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This report was prepared as part of Office of Naval Research Contract No. Nonr-1034(00), Biological Sciences Division.

INTRODUCTION

ONE YEAR before the official birth of the tissue culture technique (1906), Aldershoff and Boers [1] noted, without appreciating the implications, the first in vitro propagation of a virus (vaccinia) when they observed the formation of Guarnieri bodies in infected corneal tissue. By 1913 the essential role of living cells in virus propagation was fully recognized by Steinhardt, Israeli, and Lambert,[2] and work in this field was well launched. Nevertheless, during the first evolutionary years of tissue culture the apparent complexities of the techniques discouraged investigators in the field of virus diseases, and it was not until Maitland and Maitland,[3] reported the growth of vaccinia virus in a simple preparation of tissue, serum, and salts that virologists were encouraged to study the filterable agents in vitro, using the techniques developed by anatomists. As a matter of fact, virologists soon learned that the tissue culture requirements for their experiments were appreciably lighter than those for the investigators interested in the painstaking recording of cellular activity in vitro, and it is probable that some of the rather casual tissue culture reports by virologists may have pained the students of classical morphologic techniques. Whatever the early record may have been, the research efforts in growing viruses in vitro have increased geometrically, and there has been a constant flow of contributions from the bacteriologic and virologic laboratories since 1928.

In an attempt to assay the status of various problems in the field and to give investigators a focal point for diffuse and sometimes controversial contributions to the literature, a critical review of the cultivation of viruses was published in 1939 (Sanders [4]). The present revision of that review has been prepared for several reasons. First, the unexpected response to the original article suggested an intense interest in the subject on the part of many types of professional workers. Second, the accumulation of much data since 1939 has been evidence of a great effort expended in the propagation of viruses during recent years. New viruses have been reported, and familiar ones have been more thoroughly studied. Furthermore, with added experience the bacteriologist has learned to appreciate the implications of his results to a new extent, and a frontal attack has been made upon the most fundamental problem in the field, the process of virus multiplication.

Tissue cultures and various types of embryonated egg preparations have been utilized for the in vitro growth of viruses. These two types of techniques have yielded important information, and, indeed, the division into two groups is merely one of convenience. So many data have been accumulated that the present report will be largely confined to the results obtained in tissue cultures. However, an attempt will be made to include the basic bibliography on embryonated egg studies. Another attempt, superficial though it must be, will be made to append the major studies in the egg work to each virus for the convenience of the reader. Detailed consideration of embryonated egg techniques and results of in vitro studies can be found in Beveridge and Burnet.[5]

1. Aldershoff, H., and Boers, C. M.: Ann. Inst. Pasteur **20**:779, 1906.
2. Steinhardt, E.; Israeli, C., and Lambert, R. A.: J. Infect. Dis. **13**:294, 1913.
3. Maitland, H. B., and Maitland, M. C.: Lancet **2**:596, 1928.
4. Sanders, M.: Arch. Path. **28**:541, 1939.
5. Beveridge, W. I. B., and Burnet, F. M.: Special Report Series No. 256, Medical Research Council, London, Her Majesty's Stationery Office, 1946.

It would hardly seem necessary, when so many agents have been studied in vitro, to have standards answering the questions, "When has a virus been cultivated?" However, the great number of contributions and the intensity of effort make it imperative to maintain a system of clarification. As a rule, it is quite obvious when a virus has or has not been cultivated. In some instances, however, the nature of the isolated agent may be controversial; material for isolation may not always be at hand, or there may be conflicting results. The standards given below are worthy of repetition to help clarify the record and to maintain an awareness of problems requiring further study.

STANDARDS FOR CULTIVATION OF VIRUSES

First, the distinction must be made between propagation and survival of the original virus inoculum. Confusion on this point is most easily avoided by initiating experiments with an inoculum of known potency and by carrying the virus through a sufficient number of egg or culture generations so that infectivity cannot be attributed to the original inoculum.

A corollary of this standard is the demonstration of an actual increase in the potency of virus by quantitative estimates. To illustrate this, examples are given in Charts 1 and 2, in which quantitative studies have been reported on a somewhat different basis for two viruses. In Chart 1, Weller and Enders [6] selected the maximum point of activity of mumps cultures as the transfer time, and an increase in virus potency in each culture generation is apparent from the beginning. In Chart 2, Sanders and Jungeblut [7] demonstrated a quantitative increase of virus by starting with less than one mouse unit in tissue culture and developing a growth curve of the Columbia SK virus by periodic titrations in mice.

Depending upon the virus under investigation, several techniques are available for quantitatively testing virus potency. The most common test is infection of the proper experimental host. However, since Hirst's observation that influenza potency could be estimated by the agglutination of red blood cells,[8] the technique has been extended to other viruses (Red blood cell agglutination techniques can be used with the following viruses: influenza A and B, swine influenza, vaccinia, ectromelia, fowl plague, Newcastle disease, mouse pneumonia, the mouse encephalomyocarditis group of viruses, mumps, and GD VII strain of Theiler's virus).[9] Like the hemagglutinin, other antigens have been demonstrated in tissue cultures; Maitland and Laing [10] demonstrated complement-fixing antigens in vaccinia cultures. Similarly, complement-fixing antigen has been demonstrated in mumps (Kunz)[11] and poliomyelitis (Svedmyr, Enders, and Holloway [12]). Newer techniques are constantly being studied to permit evaluation of virus potency, and some of the most intriguing in recent years have been those concerned with the effect of

6. Weller, T. H., and Enders, J. F.: Proc. Soc. Exper. Biol. & Med. **69**:124, 1948.
7. Sanders, M., and Jungeblut, C. W.: J Exper. Med. **75**:631, 1942.
8. Hirst, G. K.: Science **94**:22, 1941.
9. Koprowski, H.: Ann. Rev. Microbiol. **4**:261, 1950.
10. Maitland, H. B., and Laing, A. W.: J. Path. & Bact. **53**:419, 1941.
11. Kunz, L. J.: Unpublished data, in Robbins, F. C., and Enders, J. F.: Am. J. M. Sc. **220**:316, 1950.
12. Svedmyr, A.; Enders, J. F., and Holloway, A.: Proc Soc. Exper. Biol. & Med. **79**:296, 1952.

a virus on tissue culture cells. Since these methods are being used for epidemiologic and diagnostic purposes, they are discussed in detail in the section "Application of In Vitro Methods."

The second standard, concerned with the formation of inclusion bodies in culture preparations, may be considered presumptive evidence of virus propagation.

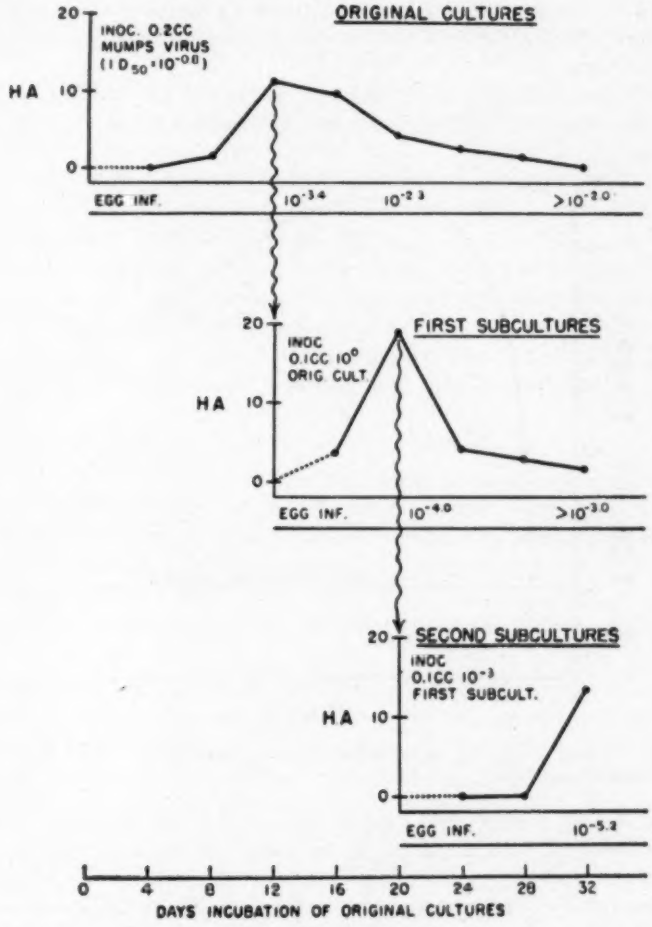

Chart 1.—Mean hemagglutinin and infectivity titers of tissue cultures inoculated with mumps virus.

This must necessarily be the least rigid of the criteria; in many instances inclusions have not been found in vivo, and it is possible to show undoubted cultivation of a virus without the presence of inclusions. However, if inclusion bodies occur, evidence for propagation becomes so much the stronger.

Third, it is desirable that confirmation of methods and results be forthcoming from one or more investigators. As will be seen later, in those viruses which have

been propagated beyond any doubt, confirmation has been consistent and frequent. It is only in cases in which propagation has not been proved that results of investigators seem to be at variance.

The fourth and final standard is concerned with control methods. Aside from the usual control procedures against bacterial invaders, a constant guard is necessary against viral contamination. The experimental animal presents three potential dangers in this respect: It may be the source of the specimen to be tested; it may be the experimental recipient of the test culture, or it may provide components for the virus culture.

Control procedures can be summarized as follows: (*a*) Routine tests for the presence of bacteria and fungi involving the usual media are done. (*b*) The virus is

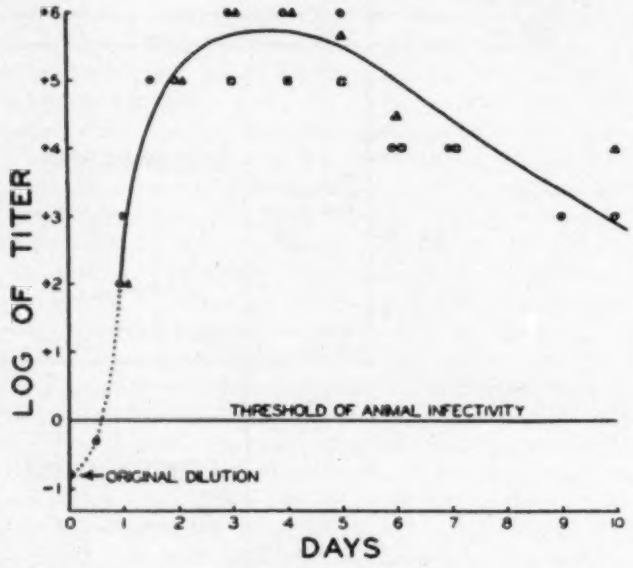

Chart 2.—Rate of propagation of the murine virus in tissue culture. Small inoculum (about 1.7 mouse-paralyzing doses).

inoculated into the routine fluid medium minus cells. This preparation should be incubated under the same conditions as the whole culture plus virus and provides the base line for the deterioration of the virus under study. It has also been suggested by Robbins and Enders [13] that a control be maintained with living cells at 4 C., at which temperature viruses presumably do not multiply. (*c*) A whole tissue culture without a virus inoculum should be maintained and tested in parallel with the standard preparation. This is the control for the presence of a contaminating virus coming from any source which provides fluid or cellular nutrition.

In applying these standards it will be seen that where an agent is propagated at will all of the criteria are met, with the exception, as the case may be, of the second, i. e., formation of inclusion bodies.

13. Robbins, F. C., and Enders, J. F.: Am. J. M. Sc. **220**:316, 1950.

MATERIALS AND METHODS

The Culture.—Since the classical observations of Harrison,[14] in 1907, on the in vitro growth of embryonic neural tissue, many variations of the basic techniques have been employed to culture many types of cells. Almost all aspects of the tissue culture work have been applied to the propagation of viruses, and virologists have added modifications of their own.

It is obviously not possible to discuss here the many facets of the tissue culture field which have been studied with scholarly intensity for 45 years, but it is desired to mention briefly several aspects of tissue culture technique which may be of interest to the student of virus-in-vitro methods. Detailed consideration of tissue culture methods may be found in extensive treatises on the subject by Fischer[15] and Parker.[16] In this regard it may be noted that fashions in tissue culture have varied from simple procedures to extremely complicated ones, the improved methodology of today lying somewhere between the extremes.

It has been customary to speak of different tissue culture techniques, but it has become apparent that there is essentially a single basic technique with minor modifications. Whether a large or small, flat or round culture receptacle is used is, after all, only a matter of the objective of the investigator. The tissues for virus growth may be maintained either as actively proliferating cells or in a more or less functional state.

Prior to 1947, workers in both the fields of tissue culture and virus diseases were accustomed to think in terms of proliferating or nonproliferating types of culture, depending upon the presence or absence of plasma. Since 1947 the fundamental contributions of Earle, Evans, Sanford, and their associates[17] have made it possible to study both the cells and the virus in a simple environment. Furthermore, the efforts of Morgan and co-workers,[16] which will be discussed later, give promise of permitting the study of the virus in controlled circumstances using synthetic media.

When Evans and Earle[18] and their associates[17a,d] showed that cellophane could be used instead of plasma for routine culture work, it was much appreciated by the workers whose technical existences had been dominated for many years by the necessity of using plasma, sometimes difficult in its collection and preparation and sometimes unpredictable in its use. That the cellophane technique was a truly effective method for obtaining proliferation of cells was seen[17b] in the observation of actual culture areas with diameters of 160 cm. after 79 days, when cultures were planted

14. Harrison, R. G.: Proc. Soc. Exper. Biol. & Med. **4**:140, 1907.

15. Fischer, A.: Tissue Culture, London, William Heinemann, Ltd., 1925; Biology of Tissue Cells, New York, Cambridge University Press, 1946.

16. Parker, R. C.: Methods of Tissue Culture, Ed. 2, New York, Paul B. Hoeber, Inc., 1950.

17. (*a*) Earle, W. R.; Evans, V. J.; Edward, M. F., and Duchesne, E.: J. Nat. Cancer Inst. **10**:291, 1949. (*b*) Earle, W. R.; Evans, V. J., and Schilling, E. L.: Ibid. **10**:943, 1950. (*c*) Sanford, K. K.; Earle, W. R.; Evans, V. J.; Waltz, H. K., and Shannon, J. E.: Ibid. **11**:773, 1951. (*d*) Schilling, E. L.; Earle, W. R., and Evans, V. J.: Ibid. **10**:883, 1950. (*e*) Shannon, J. E., and Earle, W. R.: Ibid. **12**:155, 1951.

18. Evans, V. J., and Earle, W. R.: J. Nat. Cancer Inst. **8**:103, 1947.

in 1-liter Blake bottles. In a later report these workers [19] quantitated tissue culture proliferation by counting cell nuclei in fluid aliquots, and, finally, in the most welcome report Shannon and Earle [17e] dispensed with all supporting material and planted the fibroblasts on the glass floor of the flask, with subsequent luxuriant cellular proliferation.

An even more radical alteration of tissue culture technique has been developed in Earle's laboratory and was reported in 1952.[20] Employing L-strain cells, Earle, Schilling, and Bryant [21] demonstrated rapid and unusual proliferation when cells were suspended in a medium of horse serum-embryo extract and rotated in roller drums at velocities as high as 2,400 rph instead of the usual 9 rph. The fact that adhesion of cells to the glass walls was not in evidence established the capacity of cells to proliferate without benefit of any solid substrate whatever. Thus, with rapid roller tube rotation an inoculum of 4,000,000 cells (by nucleus count) was increased to 16,000,000 within 72 hours. The potential of this technique for virus cultivation is obvious.

In the following section, the various components of virus cultures and their relationships to virus propagation will be considered briefly.

The Cell.—Selection: Although specificity of reaction between cell and virus may be considered an open question at this time, a pattern may be emerging as newer data are examined. On the one hand, selection of a gracious host cell appears to be of primary importance in some instances, but the reverse is also true, at least to the extent that some viral agents, selective in their in vivo host range, grow in vitro in many cells (normal or malignant) from apparently unrelated hosts.

Some of the evidence in favor of specific reaction between cells and virus is seen in the following examples: (*a*) Foot and mouth disease has been propagated to best effect in the presence of the most susceptible tissue (lingual mucosa) from the most susceptible host (bovine) (Frenkel [22]). Further, in this instance less satisfactory propagation of virus has been obtained in guinea pig tissue, and no growth was noted in chick tissue (Maitland and Maitland [23]). (*b*) Successful propagation of Rous sarcoma virus has occurred only in chick tissue (Sanford, Likely, Bryan, and Earle [24]). (*c*) Fowl pox virus has been grown only on chick cells and not on mouse tissue (Findlay [25]). (*d*) Virus III of rabbits has been cultivated only in the presence of rabbit tissue and not in the presence of chick cells (Ivanovics and Hyde [26]).

The recent in vitro history of poliomyelitis is an important argument against the formation of too binding a conclusion in regard to specific reactivity between cell and virus. This virus, previously considered a true neurotrope, both in vivo and in

19. Earle, W. R.; Sanford, K. K.; Evans, V. J.; Waltz, H. K., and Shannon, J. E.: J. Nat. Cancer Inst. **12**:133, 1951. Footnote 17*c*.

20. Earle, W. R.: The Preservation of Normal Tissues for Transplantation, read before the Ciba Foundation Conference, London, March 16, 1953.

21. Earle, W. R.; Schilling, E. L., and Bryant, J. C.: J. Nat. Cancer Inst., to be published.

22. Frenkel, H. S.: Am. J. Vet. Res. **11**:371, 1950.

23. Maitland, M. C., and Maitland, H. B.: J. Comp. Path. & Therap. **44**:106, 1931.

24. Sanford, K. K.; Likely, G. D.; Bryan, R. W., and Earle, W. R.: J. Nat. Cancer Inst. **12**:1317, 1952.

25. Findlay, G. M.: Brit. J. Exper. Path. **9**:28, 1928.

26. Ivanovics, G., and Hyde, R. R.: Am. J. Hyg. **23**:55, 1936.

vitro, has revealed unsuspected capacity to grow on human and monkey extraneural tissues.

It is interesting that, although human and monkey testicular tissues readily support the multiplication of poliomyelitis virus, testicular tissues of mice, guinea pigs, hamsters, rabbits, or bulls do not (Smith and co-workers,[27] Robbins and co-workers [28]). In this respect the adaptation of the virus through suckling hamsters ultimately to the egg should be noted (Roca-Garcia and co-workers,[29] Cabasso and co-workers [30]). The major portion of experimental data reviewed in the body of this report is excellent evidence of the capacity of many viral agents to grow on different tissues derived from different hosts.

Preparation: In preparing the tissue or cells for their essential role in the process of virus propagation in vitro, the technical worker does well to be guided by the principles of classical tissue culture techniques. It is generally agreed that uniformly good results are obtained when the tissues are minced with care and washed several times before transfer to the culture container. In general, maximum diameters of approximately 1 mm. for the minced tissue have permitted cellular proliferation (or maintenance of cells) with a minimum of necrosis and autolysis. It has been our practice to mince the tissue with a sharp razor on a hard surface, for example, bakelite, and after transferring the minced cells to a large watch glass to wash the suspensions three times in a salt solution which does not contain sodium bicarbonate.

State of the Cell.—The question of when a tissue culture cell is in optimal condition for virus growth has been answered in every fashion. It has been demonstrated (influenza, a prime example) that some of the agents will grow equally well in the presence of luxuriant cellular proliferation or when only a small percentage of the cells are alive, with portions of tissue undergoing autolysis. Thus, influenza will propagate with equal facility in the presence of fast-growing cells (Cushing and Morgan [31]) and in the presence of cells maintained in a functional state, as in the Maitland type of preparation (Weller and Enders [32]). This agent also grows well on the chorioallantoic membrane (Burnet [33]), where the metabolism of the cells is low. Two additional and intriguing examples of the versatility of the influenza virus are seen in the excellent yields of virus obtained in deembryonated eggs where only a single layer of chorioallantoic cells is left (Bernkopf [34]) and in its propagation in tissues of dead chick embryos when incubation is carried out for a sufficiently long period at 35 C.[35] This work is based on the report of Bucciante,[35b] who showed that living cells were present in 3-day-old embryos kept at 4 C. for 24 hours, then at

27. Smith, W. M.; Chambers, V. C., and Evans, C. A.: Proc. Soc. Exper. Biol. & Med. **76**:696, 1951.

28. Robbins, F. C.; Weller, T. H., and Enders, J. F.: J. Immunol. **69**:673, 1952.

29. Roca-Garcia, M.; Moyer, A. W., and Cox, H. R.: Proc. Soc. Exper. Biol. & Med. **81**:519, 1952.

30. Cabasso, V. J.; Stebbins, M.; Dutcher, R. M.; Moyer, A. W., and Cox, H. R.: Proc. Soc. Exper. Biol. & Med. **81**:525, 1952.

31. Cushing, R. T., and Morgan, H. R.: Proc. Soc. Exper. Biol. & Med. **79**:497, 1952.

32. Weller, T. H., and Enders, J. F.: Proc. Exper. Biol. & Med. **69**:124, 1948.

33. Burnet, F. M.: M. J. Australia **2**:687, 1935.

34. Bernkopf, H.: Proc. Soc. Exper. Biol. & Med. **72**:680, 1949; J. Immunol. **65**:571, 1950.

35. (*a*) Lahelle, O., and Horsfall, F. L.: Proc. Soc. Exper. Biol. & Med. **70**:547. 1949. (*b*) Bucciante, L.: Arch. Zellforsch. **11**:397, 1931.

room temperature for 7 days, and finally at 37 C. for 16 days. The embryos were of course dead, but surviving cells were present.

Further, on the matter of the state of the host cell, Maitland and Maitland [23] noted that the amount of foot-and-mouth-virus growth was independent of the amount of tissue growth. Hallauer [36] maintained that he was able to propagate the virus of fowl plague for 30 culture generations without tissue growth at 30 C., and, according to Magill and Francis,[37] too small or too large amounts of living cells may be detrimental to virus increase. Hecke [38] found approximately the same rate of virus multiplication when 1 or 10 pieces of tissue were placed in cultures; when 20 pieces were used, the virus increased at a faster rate.

Ratio of Tissue to Fluid.—In general, the ratio of tissue to fluid components of the culture should be considered in the light of the most favorable one for the cell. Parker [16] goes one step farther and states that, when tissue cultures are used for cultivation of viruses, the ratio between medium and tissue should be greater than under normal conditions. This is in agreement with our experience in using serum ultrafiltrate (Simms and Sanders [39]). Simms, who studied the ultrafiltrate for growing tissues, recommended a ratio of 1:100, whereas, in applying the technique to virus propagation, Sanders found a ratio of 1:300 or 1:400 more satisfactory. More recently, other investigators interested in the growth of infectious hepatitis (Henle and co-workers [40]) and poliomyelitis viruses (Syverton and Scherer [41]) in serum ultrafiltrate have had good results with ratios of 1:100 to 1:500.

That the foregoing is by no means an absolute rule is seen from the results of Li and Rivers,[42] who grew vaccinia in Tyrode's solution with a 1:50 ratio in small cultures and an approximate ratio of 1:7 in large cultures, i. e., 2 to 4 gm. of tissue to 15 cc. of Tyrode's solution.

Fluid Components.—Salt Solutions: The primary function of a salt solution is to provide a diluent containing essential salts in physiologic amounts. As will be seen later, tissues not only survive but also proliferate to some extent in the presence of salt solutions alone. Virus propagation may also occur in such a simple environment.

Tyrode's solution has been most frequently used in tissue culture work associated with viruses. In recent years, other salt solutions have come to the fore, but they may be considered modifications of the formula for Tyrode's solution (Parker [16]) given as follows: sodium chloride (NaCl) 8.00 gm., potassium chloride (KCl) 0.20 gm., calcium chloride ($CaCl_2$) 0.20 gm., magnesium chloride ($MgCl_2 \cdot 6H_2O$) 0.10 gm., sodium acid phosphate ($NaH_2PO_4 \cdot H_2O$) 0.05 gm., sodium bicarbonate ($NaHCO_3$) 1.00 gm., glucose 1.00 gm., water (glass-distilled) to make 1,000 cc., freezing point —0.62 C.

36. Hallauer, C.: Ztschr. Hyg. **113**:61, 1931.
37. Magill, T. P., and Francis, T.: J. Exper. Med. **63**:803, 1936.
38. Hecke, F.: Abstracted, Zentralbl. Bakt. (Abt. 1) **126**:93, 1932.
39. Simms, H. S., and Sanders, M.: Arch. Path. **33**:619, 1942.
40. Henle, W.; Harris, S.; Henle, G.; Harris, N. T.; Drake, N. E.; Mangold, F., and Stokes, J.: J. Exper. Med. **92**:271, 1950.
41. Syverton, J. T., and Scherer, W. F.: J. Exper. Med. **96**:355, 1952.
42. Li, C. P., and Rivers, T. M.: J. Exper. Med. **52**:465, 1930.

According to Parker, only reagent salts should be used, and it may be noted that cloudiness and high alkalinity may be avoided by placing about 850 cc. of triple-distilled water in a 1,000 cc. graduate and adding the ingredients in the order listed. The chemicals are thus dissolved completely and distributed throughout the entire volume. When all ingredients have been dissolved, water is added to make 1,000 cc. The pH should be 7.4 to 7.8. Sterilization is carried out by passing the solution through a Berkefeld or Seitz filter. Tyrode's solution cannot be sterilized by heating because of the presence of bicarbonate.

A convenient balanced salt solution with a somewhat higher concentration of phosphate and a lower concentration of calcium than in Tyrode's solution is to be found in Simms' solution (Simms and Sanders [39]). It is made up in two concentrated solutions. Fifty milliliters of each solution is added to 900 ml. of glass-distilled water. The use of concentrated solutions is a convenience, since in making up relatively small quantities of concentrates a large store of salt solution is easily made. The formula for Simms' salt solution is given as follows:

	After Dilution, Gm. per Liter	Mother Solution Concentrate, Gm. per Liter
Solution A		
Sodium chloride ($NaCl$)	8.800	160.00
Potassium chloride (KCl)	0.200	4.00
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	0.147	2.94
Magnesium chloride ($MgCl_2 \cdot 6H_2O$)	0.203	4.06
Solution B		
Sodium bicarbonate ($NaHCO_3$)	1.010	20.20
Disodium phosphate (Na_2HPO_4)	0.213	4.26
Dextrose	1.000	20.00
Phenol red	0.050	1.00

Solution A is autoclaved. Solution B is filtered through a sintered glass filter and kept stoppered in the refrigerator. Fifty milliliters of Solution A is added to 900 ml. of glass-distilled water. The whole is then autoclaved. Fifty millimeters of Solution B is added after autoclaving.

Fifteen years' experience with Simms' salt solution have shown that tissues are maintained in it to a good degree, that its pH remains quite constant, and that it can be used satisfactorily as a diluent for tissue extract and serum. The presence of phenol red as an indicator is useful in permitting observations of pH at all times. It should be noted that the formula for Simms' solution in Parker [16] is not complete, since the glucose has been omitted.

Other modifications of Tyrode's salt solution have been popular and carry the names of their originators. In actuality, it is possible that too much attention has been paid to small differences in the formulae; however, that is a matter of opinion. As Parker has noted,[16] ions which may be antagonistic "to one another, in relation to one biological process, may not be antagonistic in relation to another biological process."

Earle's solution (Earle [43]) contains more bicarbonate than the original Tyrode solution and has been considered as having a higher buffering capacity.

43. Earle, W. R.: J. Nat. Cancer Inst. **4**:165, 1943.

Hanks' salt solution (Hanks and Wallace [44]) and Gey's solution (Gey [45]) have a higher potassium and a lower calcium content than Tyrode's solution.

Tissue Extracts.—Tissue extracts made from whole embryos or from organs (particularly the spleen) have been added to cultures to provide essential growth substances. The attitude of investigators toward embryonic extracts has varied from the extremely favorable one of Carrel [46] to that of Simms,[47] who maintained that embryonic and spleen extracts contain substances inhibiting cellular growth. Carrel and his co-workers contended that embryonic extract has the power of promoting cell growth and that only through the addition of such extracts was continuous tissue culture possible. Whether or not this is true in maintaining growth of tissues or cells, the fact is clear that in the propagation of viruses serial cultivation has in many instances not been hindered by the omission of tissue extracts. Furthermore, the recent work by Sanford, Earle, and their associates [48] in regard to the use of ultrafiltrates of embryonic extracts suggests that if the extracts are essential for cell growth it is not the large molecules which are the important factors. It is interesting to compare this fact with the finding by Siddiqi and co-workers [49] that low-molecular-weight compounds containing phosphorus and nitrogen from the medium can be excluded as contributors to the virus substance (phage). If this is generally true, the ultrafiltrates may be fundamentally important to the cell nutrition and thus only indirecly to the virus.

Plasma has been the classical material for providing a permeable coagulum and for keeping tissues stationary in hanging drop and Carrel flask preparations. Homologous plasma has been commonly used but is not considered essential, particularly if homologous serum is also added. In view of the previously mentioned reports of Earle and his co-workers, the essential role of plasma appears to be questionable, and it is probable that this material will not be used in appreciable amounts in the future.

Serum has frequently been added in tissue cultures used for growing viruses. In the case of yellow fever, it was stated that serum was important for virus propagation (Haagen [50]). However, the later work of Findlay and MacCallum [51] and Fox [52] showed that serum was not essential. Since serum may be a transmitter of virus disease, for example, infectious hepatitis, it is important to determine clearly whether such material is or is not essential for virus growth. It should be pointed out that serum provides a nutritional factor which stimulates cellular growth.

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44. Hanks, J. H., and Wallace, R. E.: Proc. Soc. Exper. Biol. & Med. **71**:196, 1949.
45. Gey, G. O.: Bulletin of the Tissue Culture Commission, March 16, 1949.
46. Carrel, A.: J. Exper. Med. **17**:14, 1913.
47. Simms, H. S.: Science **83**:418, 1936.
48. Sanford, K. K.; Waltz, H. K.; Shannon, J. E.; Earle, W. R., and Evans, V. J.: J. Nat. Cancer Inst. **13**:121, 1952.
49. Siddiqi, M. S. H.; Kozloff, L. M.; Putnam, F. W., and Evans, E. A.: J. Biol. Chem. **199**:165, 1952.
50. Haagen, E.: Arch. Zellforsch. **15**:405, 1934.
51. Findlay, G. M., and MacCallum, F. O.: Brit. J. Exper. Path. **21**:173, 1940.
52. Fox, J. P.: Am. J. Hyg. **46**:120, 1947.

Serum Ultrafiltrate.—Serum ultrafiltrate (Simms and Sanders[89]) has had widespread use in recent years. Developed at Columbia University and tested in several viruses, this (grossly) protein-free material was found to be a convenient medium for growth of both cell and virus. The original rationale for the use of this fluid was the finding by Simms of a growth-promoting factor which may account for the ability of the ultrafiltrate to maintain cells for long periods of time without production of fat granules. This observation was utilized by Sanders to grow several viruses: lymphogranuloma venereum (Sanders[53]); Western and Eastern equine encephalomyelitis (Sanders and Molloy[54]); Col-Sk (Sanders and Jungeblut[7]), and epidemic keratoconjunctivitis (Sanders[55]).

Synthetic Media.—Notes on Medium No. 199: The importance of having a reproducible and known synthetic medium is so great that many investigators have made intensive efforts to produce such material. The implications of knowing the precise requirements of cellular growth have been sufficiently staggering to discourage many of the workers.

A complete and interesting history of the development of synthetic media from 1911 (Lewis and Lewis[56]) to the most recent formula, No. 199 (Morgan, Morton, and Parker) is given by Morgan (Parker[16]). Although Morgan points out that formula No. 199 is considered by the original investigators to be a basal solution for the growth of cells, it should be kept in mind that the growth requirements of viruses as related to the host cell may be less rigorous than those necessary for cellular activity. In other words, there is a possibility that mixture No. 199, incomplete as a synthetic cell propagator, may be more useful in its present form for growing viruses than for classical tissue culture. Already reports have been made on the usefulness of mixture No. 199 in growing viruses (Franklin and co-workers,[57] Thicke and co-workers,[58] Wood and co-workers,[59] Duncan and co-workers[60]).

Bovine Amniotic Fluid.—Early in 1953, Enders[61] noted that bovine amniotic fluid could be used to advantage as a medium for the cultivation of tissues and viruses. He stated that the bovine amniotic fluid was easly obtained from intact gravid uteri under sterile conditions and in amounts varying from 0.5 to 1.5 cc. per uterus. According to this investigator, the bovine amniotic fluid contains a growth-stimulating factor or factors which are best utilized when they are mixed with 5 to 10% beef embryonic extract or a similar concentration of horse serum. In his opinion this combination is superior to balanced salt-serum ultrafiltrate mixtures as judged by proliferation of tissue and virus yields. In this first report,

53. Sanders, M.: J. Exper. Med. **71**:113, 1940.

54. Sanders, M., and Molloy, E.: Proc. Soc. Exper. Biol. & Med. **45**:327, 1940.

55. Sanders, M.: Arch. Ophth. **28**:581, 1942.

56. Lewis, M. R., and Lewis, W. H.: Anat. Rec. **5**:277, 1911.

57. Franklin, A. E.; Duncan, D.; Wood, W., and Rhodes, A. J.: (*a*) Proc. Soc. Exper. Biol. & Med. **79**:715, 1952; (*b*) Canad. J. M. Sc. **31**:64, 1953.

58. Thicke, J. C.; Duncan, D.; Wood, W.; Franklin, A. E., and Rhodes, A. J.: Canad. J. M. Sc. **30**:231, 1952.

59. Wood, W.; Franklin, A. E.; Clark, E. M.; Duncan, D., and Rhodes, A. J.: Proc. Soc. Exper. Biol. & Med. **81**:434, 1952.

60. Duncan, D.; Franklin, A. E.; Wood, W., and Rhodes, A. J.: Canad. J. M. Sc. **31**:75, 1953.

61. Enders, J. F.: Proc. Soc. Exper. Biol. & Med. **82**:100, 1953.

poliomyelitis, herpes simplex, a strain of coxsackie, and four other unknown viruses have successfully propagated in a mixture consisting of 90% bovine amniotic fluid, 5% beef embryonic extract, and 5% horse serum. This medium may also be used for the study of cytopathogenic changes and therefore for the isolation of viruses, as well as neutralization studies.

VIRUS MULTIPLICATION

The most important aspect of virus study is the question of how these agents propagate, and a problem so fundamental in its general biologic implications must inevitably attract considerable scientific attention. In the hundreds of pages published on this subject are to be found facts derived from ingenious experiments planned and carried out by investigators representing every phase of biologic training. Little more can be done at this point than the presentation of opinion and a representative bibliography. In regard to the latter, it is emphasized that, while the data in this section represent the work of many investigators, a significant portion of the material is derived from two symposia [62] which appear to us to be the most comprehensive and recent discussions of the subject. For clarification of the role of the numerous investigators who made the original contributions, the reader is referred to source papers. In one instance [62a] the symposium is limited to bacterial viruses; in the other,[62b] both animal and bacterial viruses are discussed.

To begin, it must be noted that a completely lucid and precise explanation of the course of events during virus multiplication is not available at this time. In our present state of knowledge, certain theories have been formed which may be altered in the future but which provide a framework for thought and experimentation.

Although the present communication is concerned with propagation of animal viruses, the problem cannot be considered without recognition of the work in bacterial viruses, since much of the experimental data compiled on the subject of virus multiplication have been assembled in the latter field. The reason for this is eloquently stated by Putnam,[63] "With animal viruses, systemic effects, inflammatory processes, and the complexity of the tissues, all obscure profound intracellular changes . . . . by contrast the bacteriophages offer special advantages for study of the intimate association and interaction of host cell and virus because of nonpathogenicity, ease and economy of production, simplicity and precision of the assay, but most of all because of their capacity for growth in single cycles in a unique cellular host which can be cultured in a simple synthetic medium." While there is no gainsaying the superiority of phage investigation in the field of virus multiplication, it should be remembered that these data have been obtained in

62. (*a*) The Chemistry and Physiology of the Nucleus, Brookhaven National Laboratory Symposium, New York, Academic Press, Inc., 1952. (*b*) The Nature of Virus Multiplication, Oxford University Symposium, Society for General Microbiology, London, Oxford University Press, 1952. (*c*) The authors wish to express their sincere thanks to Dr. H. B. Maitland, of the University of Manchester, who loaned one of us the galley proof of the symposium on "The Nature of Virus Multiplication" and to Dr. W. C. Van Heyningen, of Oxford University, who graciously permitted study and reference to the uncorrected proof of the symposium prior to publication.

63. Putnam, F. W., in The Chemistry and Physiology of the Nucleus,[62a] p. 346.

limited experimental circumstances, and the conclusions should be viewed in that light. However, with the limitation of method kept in mind, it appears reasonably certain that common denominators are present in the animal and bacterial viruses.

Investigators are in agreement on the following points related to virus propagation. First, as has been frequently stated, all viruses do not have enzymes commonly associated with metabolic processes. Second, the process of virus multiplication appears to be intracellular in nature. Third, if reproduction is occurring, the virus must obtain substance and energy. Fourth, the reproductive procedure has genetic continuity. These are general and integrated but not necessarily sequential points, and there are other facets of the problem, obscure in detail because of the limitation of methodology in protein and enzyme chemistry.

In actuality there are few aspects of the problem of virus multiplication which are not controversial. Even the generally accepted fact that there are no enzymes available to the virus is accepted with qualifications, the point being made by Cohen [64] that biochemists cannot be certain of the absence of important enzymes related to protein organization since there are no tests for such enzymes. In this sense, the psittacosis-lymphogranuloma group is not included in the virus classification. Similarly, Bauer [65] emphasizes that in the animal viruses only vaccinia and influenza have been properly investigated, and even in these instances examination has been incomplete in his opinion. However, Bauer expresses an unwillingness to accept as a certainty the absence of enzymes until adequate purification methods have been worked out for all viruses and until they have been properly studied. With these exceptions, the basic assumption is followed that the true virus represents the ultimate in parasitism and is correspondingly dependent upon external factors for its nourishment and energy.

The second assumption, that virus multiplication occurs only in the presence of intact cells, has substance in two directions: (*a*) the fact that animal viruses have heretofore required the presence of at least a few intact living cells for multiplication, (*b*) visual experience in the field of bacterial viruses where it has been possible to follow to some extent the sequence of events in the propagation of the filterable agents. As a corollary of this assumption, virus particles presumably are absorbed onto the cellular surface. Whether or not this occurs by ionic forces (Puck and co-workers [66]), it is agreed that the virus particle disintegrates in a matter of minutes (in the case of phage) either during virus penetration of the cell or after it is inside the cell (Kozloff [67]). It is quite possible that a similar sequence of events occurs when the infecting particle is in contact with the cellular membrane in the case of vaccinia (Bauer [68]), influenza (Henle and co-workers [69]), and psittacosis (Hoyle [70]; Girardi, Allen, and Sigel [71]), since there is evidence

64. Cohen, S. S.: Ann. New York Acad. Sc. **54**:902, 1952.
65. Bauer, D. J., in The Nature of Virus Multiplication,[62b] Chap. 3, p. 11.
66. Puck, T. T.; Garen, A., and Cline, J.: J. Exper. Med. **93**:65, 1951.
67. Kozloff, L. M., in The Chemistry and Physiology of the Nucleus,[62a] p. 367.
68. Bauer, D. J.: Nature, London **164**:767, 1949.
69. Henle, W.; Henle, G., and Rosenberg, E. B.: J. Exper. Med. **86**:423, 1947.
70. Hoyle, L.: Brit. J. Exper. Path. **29**:390, 1948.
71. Girardi, A. J.; Allen, E. G., and Sigel, M. M.: J. Exper. Med. **96**:233, 1952.

of active virus disintegration during the early steps in the reproductive process in these viruses.

Once the virus particle has entered the cell, the reproductive process is temporarily hidden from the view of the investigator in both the bacterial and the animal viruses. In the case of the phage, Lowff [72] has called this the "dark period," and in influenza Hoyle has pointed out that the elementary body also disappears for about two hours without any evidence of the presence of virus in the cell. For a complete review of the sequence of events in the propagation of influenza virus in eggs, the reader is referred to Burnet,[73] Hoyle, [74] Henle,[75] Henle and Henle,[76] and Burnet and Lind.[77] In spite of the so-called dark period, it is thought that the following events occur. The virus particle probably disintegrates and may enter in a rather nonspecific fashion into the synthesis of nucleoprotein in the progeny (Kozloff [67]), at the same time transmitting genetic structures or continuity. As Chantrenne [78] describes the virus cell activity, ". . . the introduction of the virus into a receptive cell causes it to produce a group of proteins that the non-infected cell would never have produced, as if the presence of a virus particle had caused the cell mechanism to reduplicate that particle many fold."

The nature and course of the protein synthesis, which is a part of virus propagation, is a complex field in itself, with many technical limitations and pitfalls. For present purposes, a brief summary of a few somewhat contradictory opinions is given with an emphatic note of caution that the host-virus system providing the chemical information has, for the most part, been a bacterial cell and phage.

(*a*) Ribonucleic acid is involved in the self-duplicating processes (Chantrenne [78]) and growth of a virus (phage) within a cell is accompanied by a rapid increase in ribonucleic acid content (Brachet [79]).

(*b*) Desoxyribonucleic acid is also organized into the new virus particles (reviewed by Siddiqi, Kozloff, Putnam, and Evans [49]), about two-thirds of the desoxyribonucleic acid being supplied from components of the media.

(*c*) After infection, presumably most of the virus protein is synthesized de novo from the ammonium lactate of the medium.[49] However, Hoyle [80] states that virus synthesis in cells is carried out at the expense of normal protein synthesis.

(*d*) According to Weed and Cohen,[81] host-cell pyrimidines are utilized directly for viral synthesis. Sixty to ninety per cent of virus phosphorus comes from host nucleic acid. A similar percent of nitrogen in virus nucleic acid stems from host nucleoprotein. However, only about 40% of protein nitrogen of virus comes from host nitrogen.

72. Lwoff, A., in The Nature of Virus Multiplication,[62b] Chap. 7, p. 105.
73. Burnet, F. M.: J. Gen. Microbiol. **5**:46, 1951; ibid. **5**:54, 1951.
74. Hoyle, L.: Brit. J. Exper. Path. **29**:390, 1948; J. Hyg. **48**:277, 1950.
75. Henle, W.: J. Exper. Med. **90**:1, 1949; ibid. **90**:13, 1949.
76. Henle, W., and Henle, G.: J. Exper. Med. **90**:23, 1949.
77. Burnet, F. M., and Lind, P. E.: J. Gen. Microbiol. **5**:59, 1951; ibid. **5**:67, 1951.
78. Chantrenne, H., in The Nature of Virus Multiplication,[62b] Chap. 1, p. 1.
79. Brachet, J.: Expos. ann. Biochim. méd. **12**:1, 1951.
80. Hoyle, L., in The Nature of Virus Multiplication, [62b] Chap. 2.
81. Weed, L. L., Cohen, S. S.: J. Biol. Chem. **192**:693, 1951

(*e*) Host purines are used intact for synthesis of virus nucleic acid (Putnam[68]).

From even a superficial consideration of opinions concerning virus propagation, it is obvious why investigators in this problem consider it at the molecular level. And Bawden's idea that the propagation of a virus is to be compared more with the reproduction of cell constituents than to the reproduction of a cell or of an organism is understandable, as noted by Lwoff.[72] It has been noted in this respect (Sanders[82]) that "the parts of viruses responsible for initiating replication are probably smaller than the infectious units," and ". . . we know practically nothing of the size and physical and chemical properties of viruses in the state when they are actually multiplying."

Interference Phenomenon.—The problem of virus multiplication cannot be considered without appreciating the importance of that phenomenon known as interference. An effective description of the process has been given by Lennette and Koprowski,[83] "The antagonistic action between two viruses which results in the dominance of one and confers on the host a transitory resistance to superinfection with the other." When two viral agents attack the same cell, interference is by no means the only result. There are several examples of dual virus infections in the same cell.[84]

The process of interference has interested many investigators who have studied the mechanism by means of in vivo and in vitro techniques, using active and inactive forms of virus. The result has been the development of a broad and intricate literature, almost a field in itself. The entire subject of interference has been completely reviewed through 1949 by Henle[84a] and more recently by Lennette.[84b]

The interference phenomenon can be demonstrated by injecting two viruses simultaneously or by injecting the interfering agent before or after the virus to be excluded (Henle[84a]). In each instance the agents may be related or unrelated immunologically. Of fundamental importance to the demonstration of the phenomenon in all instances are the factors of timing, dosage and route of inoculation.

As Henle pointed out, interference can be evaluated by "survival or death of the test animal . . ., or by an increase in survival time . . ., by absence or presence of pathognomonic signs and lesions . . ., or of hemagglutinin . . ., or by a decrease in the severity of lesions," as well as by other techniques.

APPLICATIONS OF TISSUE CULTURE TECHNIQUES

The relatively controlled study and manipulation of viruses in tissue cultures make possible certain important applications. These include investigation of virus propagation, investigation of viral epidemiologic and diagnostic problems, investigation of virus metabolic effects, and production of antiviral vaccines.

The role of tissue culture methods in the study of the problem of virus propagation has been treated, and the status of tissue culture vaccines is to be found in the sections on the individual viruses (foot and mouth disease, poliomyelitis, vaccinia, yellow fever, and rabies).

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82. Sanders, F. K., in The Nature of Virus Multiplication,[62b] Chap. 15, p. 119.

83. Lennette, E. H., and Koprowski, H.: J. Exper. Med. **83**:195, 1946.

84. (*a*) Henle, W.: J. Immunol. **64**:203, 1950. (*b*) Lennette, E. H.: Ann. Rev. Microbiol. **5**:277, 1951.

Epidemiology and Diagnosis (Cytopathogenic Phenomenon).—The advantages of tissue culture techniques to the investigator of viral epidemiologic or diagnostic problems can be seen in certain primary isolations in tissue cultures, for example, epidemic keratoconjunctivitis (Sanders[55]) and infectious hepatitis (Henle and co-workers[40]). Embryonated egg preparations may also be used for primary isolation of viruses (French[85]). Whether such studies are possible because the culture system permits handling of large inocula during a crucial, unstable period of host adaptations (from the human to the mouse) or whether the culture is peculiarly suited for the adaptive process and the development of mutants remains to be seen. Certainly tissue culture methods provide the epidemiologist and diagnostician with a catholic, relatively simple controlled biological system which can be maintained economically and conveniently.

Recent developments have created a new emphasis in virus tissue culture work. Where formerly the tissue culture system has been dependent on animal hosts for quantitative measurements of virus potency, now the tissue culture is being developed into a self-sufficient bioassay system.

The term "cytopathogenic phenomenon" is original with Enders,[86] but the phenomenon he observed in poliomyelitis-infected tissue culture has been demonstrated by several investigators in other viruses where visible changes in the cultures have been associated with the activity of the virus under study.

Cytologic changes in cultured tissues infected with virus III were observed by Andrewes,[87] who was able to titrate serum antibodies in tissue culture using the absence of inclusion bodies as the criterion for neutralization. Plotz and Ephrussi,[88] in 1933, noted the lack of migration or peripheral growth of cells infected with fowl plague virus. In 1936, Ivanovics and Hyde[26] observed extensive pathologic changes in cultures of various rabbit tissues infected with virus III, and seven years later Huang[89] reported that when fragments of tissue previously incubated with Western equine encephalomyelitis were plasma patched, they did not coalesce as did the control tissue pieces. The same phenomenon could not be demonstrated with St. Louis encephalitis or Col-SK, and Huang,[90] instead, utilized the lack of pH change in infected cultures to measure virus potency. He was thus able to titrate virus and neutralizing antibodies by in vitro methods.

The cytopathogenic phenomenon assumed its proper importance with the work of Enders, Robbins, and Weller,[91] on the poliomyelitis virus. This virus, when propagated in tissue cultures, was observed to produce degeneration of cellular components, inhibition of cellular migration, and a reduction of glycolysis evidenced by a lack of pH change. As was to be expected, this technique has been utilized for epidemiologic surveys. During the course of such studies, strains of Coxsackie virus have been isolated (Riordan and co-workers,[92] Robbins and co-

85. French, E. L.: M. J. Australia **1**:100, 1952.

86. Enders, J. F.: Personal communication to the author.

87. Andrewes, C. H.: Brit. J. Exper. Path. **10**:188, 1929.

88. Plotz, H., and Ephrussi, B.: (*a*) Compt. rend Soc. biol. **112**:525, 1933; (*b*) ibid. **113**: 711, 1933.

89. Huang, C. H.: J. Exper. Med. **78**:111, 1943.

90. Huang, C. H.: Proc. Soc. Exper. Biol. & Med. **54**:158, 1943.

91. Robbins, F. C.; Enders, J. F., and Weller, T. H.: Proc. Soc. Exper. Biol. & Med. **75**:370, 1950. Footnote 28.

92. Riordan, J. T.; Ledinko, N., and Melnick, J. L.: Am. J. Hyg. **55**:339, 1952.

workers,[93] Melnick and Agren [94]) with a few of the strains exhibiting cytopathogenic capacities. Unclassified cytopathogenic viruses have also been occasionally isolated from stools in roller tube cultures of monkey testicular [95] and human tissues.[93] Since these viral agents are not virulent for monkeys, mice, or hamsters, the tissue culture technique is the only method presently available for their isolation. The role of these agents in human disease is not known.

Czerey-Pechany, Belady, and Ivanovics [96] have extended the observation of cytopathogenic effects to the virus of pseudorabies and have utilized the phenomenon for the titration of pseudorabies virus and for the measurement of neutralization indices of specific antiserum.

Metabolic Studies.—It is obvious that tissue culture techniques are particularly suited to the investigation of factors influencing virus growth in isolated tissue systems and also the investigation of the metabolism of cultured tissues infected with virus.

Much of the work concerned with virus growth factors has been carried out in tissue cultures with metabolites or their antagonists. It has been determined (Morgan [97]) that pteroylglutamic acid and/or citrovorum factor are essential growth factors for psittacosis, whereas vitamin B_{12} is not. Other results [97a] suggest that sulfonamide interferes with the use of *p*-aminobenzoic acid by psittacosis in its synthesis of pteroylglutamic acid, which it requires for growth, and that pteroic acid is an intermediate step in this synthesis of pteroylglutamic acid. However, through the use of analogues [97b] it has been found that pteroylglutamic acid is also an essential metabolite for meningopneumonitis, which is not sulfonamide sensitive. Pteroylglutamic acid may, therefore, well be essential for the growth of all viruses of the psittacosis-lymphogranuloma group. Adenine, guanine, and uracil are also essential growth factors for psittacosis.[98] It is probable that these substances play an important role in the synthesis of virus desoxyribonucleic and ribonucleic acids. Less clear-cut results have been obtained in studies of the effects of certain enzyme inhibitors on psittacosis virus and on infected tissue (Burney and Golub [99]).

Several outstanding differences between the growth requirements of the psittacosis group and the smaller viruses have been noted. Vaccinia, for example, is not significantly inhibited by folic acid antagonists (Thompson and co-workers [100]) and is somewhat stimulated by sulfadiazine (Thompson [101]).

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93. Robbins, F. C.; Enders, J. F.; Weller, T. H., and Florentino, G. L.: Am. J. Hyg. **54**: 286, 1951.

94. Melnick, J. L., and Agren, K.: Proc. Soc. Exper. Biol. & Med. **81**:621, 1952.

95. Footnote 92. Footnote 94.

96. Czerey-Pechany, E.; Belady, I., and Ivanovics, G.: Acta physiol. hung. **2**:229, 1951.

97. (*a*) Morgan, H. R.: J. Exper. Med. **88**:285, 1948; (*b*) ibid. **95**:269, 1952.

98. Morgan, H. R.: J. Exper. Med. **95**:277, 1952.

99. Burney, T. E., and Golub, O. J.: J. Immunol. **60**:213, 1948.

100. Thompson, R. L.; Wilkin, M. L.; Hitchings, G. H.; Elion, G. B.; Falco, E. A., and Russell, P. B.: Science **110**:454, 1949.

101. Thompson, R. L.: J. Immunol. **55**:345, 1947.

Certain naturally occurring amino acids have been found essential for the multiplication of vaccinia,[102] poliomyelitis (Brown,[103] Brown and Ackermann[104]), Theiler's GD VII (Pearson and co-workers[105]), and influenza viruses (Ackermann[106]). A number of purines and pyrimidines are also necessary for the growth of vaccinia,[107] poliomyelitis (Brown[103]), and Russian spring-summer encephalitis viruses (Friend[108]).

In analogue studies, most vitamins have been found nonessential for poliomyelitis virus growth.[103] However, a biotin analogue was demonstrated to inhibit the multiplication of this virus.[103]

It is interesting to note that abnormally high concentrations of some amino acids, purines, and pyrimidines will inhibit the multiplication of poliomyelitis (Brown[103]), Theiler's GD VII (Pearson and co-workers[105]), influenza (Eaton and co-workers[109]), and mumps viruses (Ruffilli and Addis[110]). In addition, with carefully controlled tissue cultures, certain antiviral chemical groupings similar to those occurring in chloramphenicol have been demonstrated (Thompson and co-workers[111]). Other studies concerned with the virus inhibitory action of compounds have been reported. For example, Thompson and his associates[112] found a medial lethal dose difference of 3.7 logs between control and treated vaccinia tissue cultures when 1 γ per milliliter of benzaldehyde thiosemicarbazone was added to the culture medium. Similarly, Ackermann[113] found that a 5×10^{-4} molar concentration of *p*-aminosulfonic acid markedly inhibited the propagation of PR8 influenza virus.

Bauer[65] has competently reviewed the important work on virus metabolism, and the reader is directed to his paper for a discussion of the problem. It is sufficient for the purposes of the present review to repeat a few general considerations. In Bauer's opinion, "the results of respiration studies have so far yielded little information," but "much more progress has been made in studies of individual enzyme reactions in tissues infected with viruses." He summarizes most of the available data tabularly and concludes that "the pattern of activation or inhibition is not the same for all viruses," and that the diversity in "what may be termed their biochemical properties . . . may be an expression of specific differences in the synthetic processes utilized in the production of viruses of different types."

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102. Thompson, R. L., and Wilkin, M. L.: Proc. Soc. Exper. Biol. & Med. **68**:434, 1948. Thompson.[101]

103. Brown, G. C.: J. Immunol. **69**:441, 1952.

104. Brown, G. C., and Ackermann, W. W.: Proc. Soc. Exper. Biol. & Med. **77**:367, 1951.

105. Pearson, H. E.; Lagerborg, D. L., and Winzler, R. J.: Proc. Soc. Exper. Biol. & Med. **79**:409, 1952.

106. Ackermann, W. W.: J. Exper. Med. **93**:337, 1951.

107. Thompson, R. L., and Coates, M. S.: J. Infect. Dis. **64**:105, 1939.

108. Friend, C.: Proc. Soc. Exper. Biol. & Med. **78**:150, 1951.

109. Eaton, M. D.; Magasanik B.; Perry, M. E., and Karibian, D.: Proc. Soc. Exper. Biol. & Med. **77**:505, 1951.

110. Ruffilli, D., and Addis, S.: Boll. Soc. ital. biol. sper. **27**:1251, 1951.

111. Thompson, R. L.; Wilkin, M. L.; Hitchings, G. H., and Russell, P. B.: Proc. Soc. Exper. Biol. & Med. **72**:169, 1949.

112. Thompson, R. L.; Price, M. L., and Minton, S. A.: Proc. Soc. Exper. Biol. & Med. **78**:11, 1951.

113. Ackermann, W. W.: Proc. Soc. Exper. Biol. & Med. **80**:362, 1952.

AVIAN ENCEPHALOMYELITIS VIRUS

Tissue Cultures.—Kligler and Olitsky [114] carried the virus of avian encepahlomyelitis through five tissue culture passages. Minced, whole chick embryo suspended in 10% chicken serum-Tyrode's solution permitted multiplication of the virus but yielded low titers (10^{-2}). Cultures of chick embryo brain did not support virus propagation, and virus growth did not occur when chicken serum was not utilized in the fluid medium. All cultures were incubated at 36.5 to 37C.

Embryonated Eggs.—Attempts to pass the virus of avian encephalomyelitis through embryonated hens' eggs by yolk sac and allantoic sac inoculation proved fruitless.[114] However, the virus survived five to six weeks in the yolk sac (Feibel [115]).

BLUE COMB DISEASE VIRUS

Embryonated Eggs.—A virus isolated from the blood stream of birds affected with the blue comb disease was passed through as many as 56 transfers on the chorioallantois of embryonated eggs (Waller [116]).

When infected egg material was inoculated into birds, suggestive symptoms were produced, but the absence of any lethal effects prompted the investigators to question the identification of the cultivated virus as the sole etiologic agent of the "so-called blue comb disease."

BLUE TONGUE DISEASE VIRUS

Embryonated Eggs.—In the first report of the culture of the virus of blue tongue disease, the workers (Mason, Coles, and Alexander [117]) were successful only when the virus was inoculated into yolk sacs of embryos derived from fowls on a deficient diet. After four passages in such eggs, normal eggs supported virus growth, and 37 passages were carried out. The necessity for using eggs from riboflavin-deficient fowls as first postulated was later obviated. However, the temperature of incubation was found to be of crucial importance (Alexander [118]). The virus could be initiated in eggs incubated at 33.6 C. but not in eggs kept at 32.1 or 35.0 C. (Alexander, Haig, and Adelaar [119]).

After 20 passages in yolk sac at 35 C. for the first 24 hours and then 32 C. until death in three to four days, the virus became attenuated, but inoculation provided solid immunity against the homologous virulent virus.[119]

BOVINE ENCEPHALOMYELITIS VIRUS

Embryonated Eggs—The bovine encephalomyelitis virus propagated in the embryonated egg after yolk sac inoculation but not after allantoic sac inoculation

114. Kligler, I. J., and Olitsky, P. K.: Proc. Soc. Exper. Biol. & Med. **43**:680, 1940.

115. Feibel, F.: Personal communication, cited by Cottral, G. E.: Ann. New York Acad. Sc. **55**:221, 1952.

116. Waller, E. F.: Science **95**:560, 1942.

117. Mason, J. H.; Coles, J. D. W. A., and Alexander, R. A.: Nature, London **145**:1022, 1940.

118. Alexander, R. A.: Onderstepoort, J. Vet. Sc. **22**:7, 1947.

119. Alexander, R. A.; Haig, D. A., and Adelaar, T. F.: Onderstepoort J. Vet. Sc. **21**:231, 1947.

(Wenner, Harshfield, Chang, and Menges,[120] McNutt[121]). Seven to 10-day embryos were killed in four to nine days.[120] The virus was present throughout the egg, in highest concentration in the yolk sac. Elementary bodies were demonstrated in emulsions and smears.

B VIRUS

Embryonated Eggs—The B virus was cultured through 30 passages on the chorioallantoic membrane (Burnet, Lush, and Jackson[122]). Embryos were killed in four to six days and exhibited focal lesions in the viscera. Microscopic lesions of the membrane were much like those of herpes simplex.

CANINE DISTEMPER VIRUS

Embryonated Eggs.—Canine distemper virus was successfully cultivated on the chorioallantoic membrane of embryonated eggs (Cabasso and Cox,[123] Haig[124]) after several attempts in other laboratories were unsuccessful (Plummer,[125] Mitscherlich,[126] Beveridge and Burnet[5]). After extended serial passage the virus lost its capacity to infect ferrets but retained its antigenicity (Cabasso and Cox[123]). Lesions did not develop on the membrane until the 10th passage, after which opaque foci became increasingly marked and numerous. Further, the chorioallantois began to present a thickened appearance. The egg-attenuated virus was used extensively as a vaccine (Cox[127]). A canine encephalitis virus immunologically similar to canine distemper was maintained for 58 serial passages on the chorioallantoic membrane after a preliminary adaptation program. The virus lost its virulence for ferrets after 23 passages but retained the capacity to protect ferrets against the virulent virus (Cabasso and Cox[128]).

CANINE HEPATITIS VIRUS

Embryonated Eggs.—The canine hepatitis virus has been demonstrated to propagate in the yolk sac of 6-day-old embryonated eggs for 11 passages (Miles, Parry, Larin, and Platt[129]). The virus subsequent to the fifth passage was lethal for 50% of the embryos by the 10th day. The embryos showed necrotic livers, but no inclusion bodies could be found.

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120. Wenner, H. A.; Harshfield, G. S.; Chang, T. W., and Menges, R. W.: Am. J. Hyg. **57**:15, 1953.

121. McNutt, S. H.: North American Vet. **23**:242, 1942.

122. Burnet, F. M.; Lush, D., and Jackson, A. V.: Australian J. Exper. Biol. & M. Sc. **17**:35, 1939.

123. Cabasso, V., and Cox, H. R.: Proc. Soc. Exper. Biol. & Med. **71**:246, 1949.

124. Haig, D. A.: J. South African Vet. M. A. **19**:73, 1948; Onderstepoort J. Vet. Sc. **23**: 149, 1948.

125. Plummer, P. J. G.: Canad. J. Compt. Med. **3**:96, 1939.

126. Mitscherlich, E.: Deutsche tierärztl. Wchnschr. **46**:497, 1938.

127. Cox, H. R.: Ann. New York Acad. Sc. **55**:236, 1952.

128. Cabasso, V., and Cox, H. R.: Cornell Vet. **42**:96, 1952.

129. Miles, J. A. R.; Parry, H. B.; Larin, N. M., and Platt, H.: Nature, London **168**:699, 1951.

COMMON COLD VIRUS

Tissue Cultures.—The greatest difficulty in evaluating attempts to propagate the virus of the common cold lies in the fact that the specific etiologic agent for a generally accepted disease has not been isolated to the satisfaction of all workers. Even when agents have been isolated, the lack of an experimental host other than the human volunteer has made the interpretation of results unsatisfactory. Positive reports on the cultivation of a coryza virus have been made by Dochez, Mills, and Kneeland,[130] who maintained the virus for as long as 17 culture generations and produced colds in human volunteers with material from final subcultures. Powell and Clowes [131] repeated this work for 31 cultures and also produced symptoms in volunteers. The preparations used in these experiments consisted of minced chick embryos and Tyrode's solution or buffered bouillon containing cysteine hydrochloride.

Embryonated Eggs.—Attempts to demonstrate propagation of the virus of the common cold in the embryonated egg have been contradictory. Several groups of investigators reported their ability to pass the virus through embryonated eggs on the chorioallantoic membrane and in the allantoic cavity (Kneeland, Mills, and Dochez [132]; Pollard and Caplovitz [133]; Topping and Atlas [134]; Ward and Proctor [135]). Other workers have been unable to obtain any positive results whatever (Beveridge and Burnet,[5] Andrewes [136]). The greatest number of transfers for which the virus remained definitely pathogenic for human volunteers infected intranasally was nine (Pollard and Caplovitz [133b]); when the ninth passage material was tested in a group of human volunteers, five out of six showed typical common cold symptoms. Normal chorioallantoic fluid had no similar effect when administered to human controls.

The same difficulty noted in tissue culture reports of the common cold prevails in the embryonated egg work. Until precise criteria for a specific disease are available, it is difficult to see how the question of the common cold propagation can be answered.

COXSACKIE VIRUS

Tissue Culture.—Slater and Syverton [137] observed propagation of a strain of Coxsackie virus (Group A, Type 4) isolated from a fecal specimen and passed through newborn mice. Brain, intestine, or muscle tissue of mice killed 48 hours before or after birth was suspended in serum ultrafiltrate, and 24 subcultures were carried out with each tissue type. The passages constituted a total dilution of

130. Dochez, A. R.; Mills, K. C., and Kneeland, Y.: Proc. Soc. Exper. Biol. & Med. **28**: 513, 1931; ibid. **29**:64, 1931; J. Exper. Med. **63**:559, 1936.

131. Powell, H. M., and Clowes, C. H. A.: Proc. Soc. Exper. Biol. & Med. **29**:332, 1931.

132. Kneeland, Y.; Mills, K. C., and Dochez, A. R.: Proc. Soc. Exper. Biol. & Med. **35**:213, 1936.

133. (*a*) Pollard, M., and Caplovitz, C. D.: Science **106**:243, 1947; (*b*) Am. J. Hyg. **47**:106, 1948.

134. Topping, N. H., and Atlas, L. T.: Science **106**:636, 1947.

135. Ward, T. G., and Proctor, D. F.: Am. J. Hyg. **52**:91, 1950.

136. Andrewes, C. H.: Lancet **1**:71, 1949.

137. Slater, E. A., and Syverton, J. T.: Proc. Soc. Exper. Biol. & Med. **74**:509, 1950.

166 [24], and it was reported that the tissue culture virus up to the 12th culture was uniformly lethal for mice at a dilution of 10^{-6}, but after the 16th culture this dilution was only 42% lethal, although all the mice were paralyzed by the dose.

Shaw [138] reported multiplication of four Group A, Type 2 strains and one Group A, Type 4 strain of Coxsackie virus in suspended cell cultures of chick embryo in serum ultrafiltrate. Three Group A, Type 4 strains and eight strains representing Types 1, 3, 5, and 6 did not give evidence of growth in this culture medium.

Stulberg, Schapira, and Eidam [139] investigated the ability of Group B, Type 1 Coxsackie virus to propagate in Porter roller flask cultures of fibroblasts. The source of the tissue was the interscapular fat pads of newborn mice, and by the third subculture fibroblasts predominated to the extent that other cell types were not microscopically detectable. The third fibroblast passage, when inoculated with $10^{2.5}$ LD_{50} showed no titer increase until the third day of incubation, and the titer reached a maximum of $10^{6.4}$ on the fifth day; whereas in the primary tissue culture, which contained both explanted host cells and outgrowing fibroblasts, the virus exhibited only a brief lag phase and attained the maximum titer by the third day. Cytopathogenic effects, as evidenced by the failure of the pH of infected cultures to drop as low as the pH of the uninoculated cultures and by increased granulation and subsequent necrosis of th cells, were noted in the two culture generations. In the case of the third fibroblast passage, cell destruction became apparent before the advent of maximum titers in the extracellular fluid. Cell-free control cultures contained no demonstrable virus after two days, and in cultures of chick embryo cells the virus was not apparent after four days of culture.

Only a few strains of Coxsackie virus were cytopathogenic for fibroblasts derived from human or monkey tissue. Riordan, Ledinko, and Melnick [92] found that 1 or 2 of 15 antigenically distinct Coxsackie viruses readily produced fibroblastic degeneration of the cellular outgrowth from monkey testicular tissue. Similarly, Melnick and Agren [94] reported that one of nine Coxsackie viruses isolated in Egypt were cytopathogenic for this tissue. Robbins, Enders, Weller, and Florentino [93] and Weller, Robbins, and Stoddard [140] also demonstrated Coxsackie cytopathogenicity in cultures of human tissues, and, in agreement with other workers, Riordan, Ledinko, and Melnick [92] and Melnick and Agren [94] noted that the Wieder strain of Coxsackie virus or antigenically related strains readily multiplied and produced cytopathogenic changes.

Embryonated Eggs.—Successful cultivation of the Coxsackie virus in embryonated eggs has not been uniformly achieved. First attempts with newly isolated virus were negative (Dalldorf, Sickles, Plager, and Gifford [141]). In another study a Type 2 virus was established in chick embryos only after a series of alternating mouse-egg passages (Huebner, Ransom, and Beeman [142]). In yet another study

138. Shaw, M.: Proc. Soc. Exper. Biol. & Med. **79**:718, 1952.

139. Stulberg, C. S.; Schapira, R., and Eidam, C. R.: Proc. Soc. Exper. Biol. & Med. **81**:642, 1952.

140. Weller, T. H.; Robbins, F. C., and Stoddard, M. B.: Fed. Proc. **11**:486, 1952.

141. Dalldorf, G.; Sickles, G. M.; Plager, H., and Gifford, R.: J. Exper. Med. **89**:567, 1949.

142. Huebner, R. J.; Ransom, S. E., and Beeman, E. A.: Pub. Health Rep. **65**:803, 1950.

of 5 strains of Group B and 24 strains of Group A Coxsackie virus, only 2 Group A, Type 2 strains could be carried through more than two egg transfers in the yolk sac (Shaw [138]). One strain carried for eight passages gave no gross evidence of infection in the egg, and the incidence of egg infectivity was noted to be low. Another strain adapted readily to propagation in the yolk sac was occasionally lethal for eggs and produced hyaline degeneration of embryonic striated muscle. A 10^{-7} dilution of the seventh passage produced no central nervous system lesions in mice. The Easton 10C strain of Coxsackie virus was passed through five embryonated eggs and exhibited a decrease in mouse virulence (Godenne and Curnen [143]).

DENGUE VIRUS

Embryonated Eggs.—Schlesinger [144] suggested that until his own experiments in 1950 the various investigators working with dengue virus had failed to identify the agent they claimed to have cultivated in the embryonated egg. Schlesinger inoculated 101st mouse brain passage virus into 5-day-old fertile eggs by perembryonic stab. Passages were performed every seven days, and 48 transfers were carried out with no loss of mouse virulence. Homogenized embryo injected into humans caused a dengue-like rash. Additional confirmatory reports are necessary before a definite conclusion can be drawn concerning the behavior of the dengue virus in eggs.

ECTROMELIA VIRUS

Tissue Culture.—Downie and McGaughey [145] cultivated the virus of ectromelia for seven culture generations in preparations of mouse embryonic tissue, mouse serum, and Tyrode's solution. The increase in potency of the final culture was estimated at $6 \times 10.^{14}$ The authors noted degeneration of cells and could demonstrate the virus in both the tissue and the fluid elements of the cultures. When serum was not added or when embryonic chick tissue replaced the mouse tissue, multiplication of the virus still occurred. Inclusion bodies were noted in explanted cells.

The same authors also demonstrated [146] that immune serum, when added to infected cultures, did not neutralize or destroy the virus, but its multiplication was hindered.

Although the evidence for propagation appears to be consistent, further confirmation with greater numbers of subcultures is desirable.

Embryonated Eggs.—The virus of ectromelia has been cultivated on the chorioallantois (Paschen,[147] Burnet and Lush,[148] Himmelweit [149]) for as many as 18 transfers.[147] With an increase in egg passages, embryo deaths occurred more frequently; ectodermal proliferation and necrosis in the form of small opaque lesions became more marked, and inclusion bodies could be observed.[148] Virus growth was apparent only in eggs incubated below 37 C. At 35 C. death of the embryos was regular. Specific hemagglutinins were demonstrated in emulsions of infected chorioallantoic membrane (Burnet and Boake.[150])

143. Godenne, M. O., and Curnen, E. C.: Proc. Soc. Exper. Biol. & Med. **81**:81, 1952.
144. Schlesinger, R. W.: Am. J. Hyg. **51**:248, 1950.
145. Downie, A. W., and McGaughey, C. A.: J. Path. & Bact. **40**:147, 1935.
146. Downie, A. W., and McGaughey, C. A.: J. Path. & Bact. **40**:297, 1935.
147. Paschen, E.: Zentralbl. Bakt. (Abt. 1) **135**:445, 1936.
148. Burnet, F. M., and Lush, D.: J. Path. & Bact. **43**:105, 1936.
149. Himmelweit, F.: Brit. J. Exper. Path. **19**:108, 1938.
150. Burnet, F. M., and Boake, W. C.: J. Immunol. **53**:1, 1946.

JAPANESE B ENCEPHALITIS VIRUS

Tissue Culture.—Successful propagation of the virus of Japanese B encephalitis was observed for 16 culture generations in the presence of embryonic chick liver (Haagen [151]) and for 40 subcultures in the presence of embryonic chick heart (Haagen and Crodel [152]). With a 1:1,000,000 dilution of the 40th culture transfer, Haagen and Crodel were able to infect three out of three mice by intracerebral inoculation. These authors also tried testicular and splenic tissue from young rabbits and mice. The cells were bathed for 15 minutes in an emulsion of infected mouse brain and then explanted into hanging drops of plasma and embryo extract. In general, adult testicle was better than spleen. No difference in the affinity of cells for the virus was noted among various embryonic tissues.

Employing suspended cell cultures of both whole chick embryo and chick embryo brain, Kawakita [153] obtained propagation of the Japanese B virus. In these cultures nutrient fluids of allantoic fluid, chick serum in Tyrode's solution, or Tyrode's solution alone gave equivalent results. The cultures were incubated at 35 C., and transfers were performed every three to seven days. Sixty-one passages were reported, and, although the mouse infectivity titer dropped from 10^{-7} to 10^{-2} the total increase of virus potency was calculated to be 10^{60}. When the virus inoculum was allowed to incubate with the mouse brain tissue for 20 minutes prior to the addition of allantoic fluid, these cultures proved sensitive to 0.5×10^{-8} dilutions of mouse brain virus.

In later experiments Kawakita and Tazaki [154] observed that high-titered Japanese-B-immune horse serum completely neutralized the virus in the tissue and fluid portions of the culture when added as late as 48 hours after virus inoculation. The tissue and the fluid in the control preparations yielded, respectively, virus titers of 10^{-5} and 10^{-4}. In view of conflicting results obtained with immune serum in some virus cultures (vaccinia, herpes simplex, and yellow fever), Kawakita suggests that the neutralization of viruses in tissue culture depends on the "quantitative relationship between the potency of the immune serum and the virus content of the tissue at the time of their meeting."

Embryonated Eggs.—The virus of Japanese B encephalitis has been cultivated on the chorioallantois (Haagen and Crodel,[152] Smith and Lennette,[155]), in the allantoic sac (Warren and Hough [156]), in the yolk sac (Howitt [157]), and in the embryo proper (Morgan, Early, and McClain [158]). The virus attained its highest titers in the embryo and was usually lethal. The virus distribution in the egg was not affected by the route of inoculation.[158] When inoculated onto the chorioallantois, this membrane became adematous, slightly opaque, and finely stippled.[155] Hemorrhage became prominent after 35 intraembryonic passages.[158] No change in viru-

151. Haagen, E.: Abstracted, Zentralbl. Bakt. (Abt. 1) **128**:96, 1937.
152. Haagen, E., and Crodel, B.: Abstracted, Zentralbl. Bakt. (Abt. 1) **142**:269, 1938.
153. Kawakita, Y.: Jap. J. Exper. Med. **17**:211, 1939.
154. Kawakita, Y., and Tazaki, T.: Jap. M. J. **1**:17, 1948.
155. Smith, M. G., and Lennette, E. H.: Proc. Soc. Exper. Biol. & Med. **41**:323, 1939.
156. Warren, J., and Hough, R. G.: Proc. Soc. Exper. Biol. & Med. **61**:109, 1946.
157. Howitt, B. F.: Proc. Soc. Exper. Biol. & Med. **62**:105, 1946.
158. Morgan, H. R.; Early, R. L., and McClain, M. E.: J. Infect. Dis. **79**:278, 1946.

lence for mice occurred after extended yolk sac culture (Koprowski and Cox [159]). Effective vaccines for human use have been prepared (Warren and Hough [156]; Warren, Smadel, and Rasmussen [160]).

ST. LOUIS ENCEPHALITIS VIRUS

Tissue Culture.—St. Louis encephalitis virus propagated in the presence of embryonic mouse brain (Syverton and Berry [161]; Harrison and Moore [162]; Schultz, Williams, and Hetherington [163]). In view of Molloy's work,[164] the uniformly low titers (10^{-2} and 10^{-3}) of the earlier investigators might well be attributed to the 37 C. temperature of incubation. Employing the same tissue but in serum ultrafiltrate-Simms' solution, Molloy [164] found the titers at room temperature to be 10^{-5}, two logs higher than the titers observed for similar cultures incubated at 37 C. In contrast, Webster and Johnson [165] reported occasional titers from St. Louis encephalitis cultures incubated at 37 C. as high as 10^{-5} after eight passages in the presence of embryonic mouse brain and rabbit serum.

When embryonic chick tissue was substituted for mouse brain,[166] propagation also occurred. Adult guinea pigs [164] and adult mouse brain,[163] however, were not satisfactory substrates for virus growth.

Pang [167] and Pang and Zia [168] reported 40 passages of St. Louis encephalitis virus on chick embryo-agar slants, following Zinsser's technique,[169] without loss of virulence for mice. Minced chick embryo liver and mouse embryo brain gave similar results. The most rapid multiplication of the virus appeared to occur during the first 48 hours of incubation, with the maximum titers being observed between five and six days after implantation. Using this technique, Pang demonstrated virus potencies of 10^{-6} for as long as 36 days of continuous culture.

Huang [90] found no difference between the proliferation of St. Louis encephalitis-infected and noninfected, plasma-patched tissue but was able to titrate the virus in vitro utilizing the St. Louis encephalitis virus' capacity to interfere with the propagation of Western equine encephalomyelitis virus in tissue culture. Sensitive in vitro titrations [170] were also accomplished on the basis of the lack of pH change in infected tissues resulting from reduced glycolysis.

Embryonated Eggs.—The virus of St. Louis encephalitis has been cultivated at will on the chorioallantoic membrane (Harrison and Moore [162]; Schultz, Williams, and Hetherington [163]; Smith [171]). The titers of virus grown on the membrane

159. Koprowski, H., and Cox, H. R.: J. Immunol. **52**:171, 1946.
160. Warren, J.; Smadel, J. E., and Rasmussen, A. F.: J. Immunol. **58**:211, 1948.
161. Syverton, J. T., and Berry, G. P.: Science **82**:596, 1935.
162. Harrison, R. W., and Moore, E.: Proc. Soc. Exper. Biol. & Med. **35**:359, 1936.
163. Schultz, E. W.; Williams, G. F., and Hetherington, A.: Proc. Soc. Exper Biol. & Med. **38**:799, 1938.
164. Molloy, E.: Proc. Soc. Exper. Biol. & Med. **44**:563, 1940.
165. Webster, L. T., and Johnson, M. S.: J. Exper. Med. **74**:489, 1941.
166. Harrison, R. W., and Moore, E.: Am. J. Path. **13**:361, 1937. Molloy.[164]
167. Pang, K. H.: Proc. Soc. Exper. Biol. & Med. **43**:755, 1940.
168. Pang, K. H., and Zia, S. H.: Chinese M. J., Supp. 3, p. 446, 1940.
169. Zinsser, H.; Fitzpatrick, F. K., and Wei, H.: J. Exper. Med. **69**:179, 1939.
170. Huang, C. H.: Proc. Soc. Exper. Biol. & Med. **54**:160, 1943.
171. Smith, M. G.: J. Infect. Dis. **72**:125, 1943.

were not as high as those for mouse brain virus, but the egg passage virus was more infectious subcutaneously per intracerebral minimum lethal dose.[171]

Cloudy proliferative lesions with central necrosis occurred in the chorioallantoic membrane in four to seven days.[162] No lesions were found in the embryo, although death was frequent, and virus could be recovered from brain, liver, and spleen. It has been possible with special staining methods to satisfactorily demonstrate the neutralizing effect of immune serum in egg preparations (Blattner and Cooke[172]).

ENCEPHALITIS VIRUSES: CALIFORNIA, ILHÉOS, MURRAY VALLEY, RUSSIAN SPRING-SUMMER, VENEZUELAN EQUINE, WEST NILE

Tissue Culture.—In the course of studying the interference phenomenon in tissue cultures, Lennette and Koprowski[173] propagated the viruses of Venezuelan equine encephalomyelitis and West Nile disease in cultures of minced chick embryo (from which the central nervous system had been removed) suspended in 10% human serum and Tyrode's solution. Using similar preparations, Koprowski and Hughes[174] obtained multiplication of the Ilhéos virus. With each of these three encephalitis viruses, particularly in the case of the Ilhéos virus, a loss of virulence for mice of 3 to 6 logs was encountered in early passages.[175] The lower titers measured by intracerebral mouse inoculation were sustained, but after extended culture generations, the West Nile[175b] and Venezuelan[176] viruses lost additional intraperitoneal virulence for young mice. This loss of virulence beyond the initial drop in titer of intraperitoneally inoculated virus could also be demonstrated in hamsters with the West Nile agent and in guinea pigs and rabbits with Venezuelan equine encephalomyelitis. Mouse embryo brain, whole mouse embryo, and whole chick embryo were all found to support the growth of the West Nile[175b] virus.

The Russian spring-summer encephalitis virus apparently multiplied readily in suspended cell cultures containing one of several types of tissue: mouse tumor (sarcoma 180 or carcinoma 1,025), mouse embryo, or chick embryo (Friend[108]). In the case of this agent, the tissue culture technique has been effectively used to evaluate the oncolytic property of brain passage or tumor virus (Moore[177]), as well as to assay the effect of a purine antimetabolite on the proliferation of the virus.[108]

On bioassay it was seen that most of the tumor tissue was rendered nonviable by Russian spring-summer encephalitis virus; nevertheless, the virus was recovered in high titer. Hence, slight tissue growth permits viral multiplication.[178]

Although the virus was not subcultured, the titer of virus in the supernatant fluid of cultures increased 100 to 1,000 times during 24 to 96 hours of incubation. After extended tumor to tumor passage the oncolytic activity of the virus increased as measured in vitro. This was not true of the strain of virus maintained by mouse brain passage in vivo.

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172. Blattner, R. J., and Cooke, J. V.: J. Infect. Dis. **70**:226, 1942.
173. Lennette, E. H., and Koprowski, H.: J. Exper. Med. **83**:195, 1946.
174. Koprowski, H., and Hughes, T. P.: J. Immunol. **54**:371, 1946.
175. Koprowski, H., and Lennette, E. H.: (*a*) J. Bact. **48**:463, 1944; (*b*) J. Exper. Med. **84**:181, 1946. (*c*) Footnote 174.
176. Koprowski, H., and Lennette, E. H.: J. Exper. Med. **84**:205, 1946.
177. Moore, A. E.: Proc. Soc. Exper. Biol. & Med. **76**:749, 1951.
178. Friend.[108] Moore.[177]

While cultivation of each of the above viral agents has been reported by only one laboratory, there is reason to believe, in view of the experience with other encephalitis agents, that confirmation would present little difficulty. This is partially borne out by the results of Toolan and Moore,[179] who observed propagation of West Nile virus (Egypt 101) in roller tube cultures of human carcinoma previously passed through irradiated rats.

Embryonated Eggs.—California encephalitis virus was propagated following chorioallantoic membrane, allantoic cavity, amniotic sac, yolk sac, vein, or embryo inoculation (Hammon, Reeves, and Sather [180]). Egg mortality was low even after 20 serial passages.

The Venezuelan equine encephalomyelitis virus was grown on the chorioallantoic membrane and in the yolk sac (Beck and Wyckoff,[181] Kubes and Rios,[182] and Koprowski and Lennette [175a]). Central nervous system changes which occurred in infected embryos were secondary to vascular involvement.

West Nile virus, when inoculated into the chorioallantoic membrane or into the yolk sac, killed the embryos in three to five days but produced no specific pathology (Watson [183]). The virus was more concentrated in the embryo than in the extra embryonic fluids or membranes.

The Ilhéos virus was cultivated in embryonated eggs by both chorioallantoic membrane and yolk sac inoculation (Koprowski and Hughes [175a]; Taylor [184]). This virus also attained its highest titers in the embryo.[174]

A Japanese B-like virus, Murray Valley encephalitis, was isolated directly onto the chorioallantoic membrane of the embryonated egg (French [85]). The virus was later shown to be highly pathogenic for mice but could not be primarily isolated in this host. This encephalitis virus produced focal lesions on the membrane which resembled those of the adapted influenza virus. Death of the infected embryos occurred within 72 hours and was accompanied by extensive hemorrhage. Other routes of inoculation, i. e., amniotic cavity, allantoic cavity, and yolk sac, gave similar results. A complement-fixing antigen was present in the chorioallantois.

EPIDEMIC KERATOCONJUNCTIVITIS VIRUS

Tissue Culture.—In 1942, several thousand cases of a heretofore unknown clinical entity suddenly appeared in the industrial centers of the east and west coasts of the United States. The disease was characterized by an acute follicular conjunctivitis, a keratitis without ulceration, regional adenopathy, and constitutional symptoms referable to the upper respiratory tract and possibly to the central nervous system. Tissue edema and watery secretions from the affected eyes were prominent signs. After an acute phase of one to three weeks the attack subsided, usually leaving a residual, fairly characteristic keratitis.

Sanders [88] first reported isolation of a virus from the conjunctival scrapings of patients in the early stages of the disease and demonstrated a specific rise in neutralizing antibodies against the experimental agent in convalescent serum of

179. Toolan, H. W., and Moore, A. E.: Proc. Soc. Exper. Biol. & Med. **79**:697, 1952.
180. Hammon, W. M.; Reeves, W. C., and Sather, G.: J. Immunol. **69**:493, 1952.
181. Beck, C. E., and Wyckoff, R. W. G.: Science **88**:530, 1938.
182. Kubes, V., and Rios, F. A.: Science **90**:20, 1939.
183. Watson, D. W.: Proc. Soc. Exper. Biol. & Med. **52**:204, 1943.
184. Taylor, R. M.: J. Immunol. **68**:473, 1952.

patients from different areas of the United States. The original workers were able to isolate the virus only when conjunctival scrapings were put into 3 ml. tissue cultures of serum ultrafiltrate and minced embryonic mouse brain. Direct inoculation of primary material from patients into mice (intracerebral inoculation) produced a transient meningoencephalitis. Tissue cultures, on the other hand, were satisfactory substrates for serial culture when the subculture inoculum consisted of ground cells plus culture fluid following six days of incubation at room temperature (18 to 23 C.).

Additional isolations of the virus from the same epidemic areas were reported by Sanders and Alexander [185] and by Sanders and co-workers.[186] In all instances the same techniques were used, and filtration through double Seitz pads and graded collodion membranes suggested that the virus was a small one, of the order of 50 μ.

Maumenee, Hayes, and Hartman [187] also succeeded in isolating a filterable agent from the eyes of patients in the eastern United States area in 1945. These investigators did not use tissue cultures but obtained transmissible agents by direct large inoculation of scrapings into the brains of rabbits. They found a close relationship between the epidemic keratoconjunctivitis and herpes simplex viruses. In the case of isolation No. 1, Maumenee and his associates reported that challenge of herpes-simplex-and epidemic-keratoconjunctivitis- (New York) immune mice with isolation No. 1 induced infection in the herpes-simplex-immune animals but not in the epidemic-keratoconjunctivitis- (New York) immune mice. The Baltimore strains of virus were lost and are not available for further testing.

Heyl, Allen, and Cheever [188] found no neutralizing antibodies against the epidemic keratoconjunctivitis virus in the gamma globulin fraction of plasma pools of human serums. In contrast, the same blood fractions neutralized herpes simplex virus. However, in 1952 both Ruchman [189] and Cheever [190] found a close immunologic relationship between St. Louis encephalitis and epidemic keratoconjunctivitis viruses. Indeed, on the basis of their reports, the two viruses could not be differentiated. But in this respect it should be noted that the titer of the epidemic keratoconjunctivitis virus had suddenly changed without apparent explanation in later mouse passage.

Braley [191] has apparently succeeded in isolating epidemic keratoconjunctivitis virus from the eyes of patients by following the original technique in tissue cultures. In March, 1953, a definitive study on the cultivation of the epidemic keratoconjunctivitis virus was reported.[192] Sezer isolated a virus from eye washings of epidemic keratoconjunctivitis patients on human cornea explanted to fertilized eggs. The agent was also grown in tissue culture, and its action in mice and in human volunteers strongly suggests that this investigator worked with an agent similar or

185. Sanders, M., and Alexander, R. C.: J. Exper. Med. **77**:71, 1943.

186. Sander, M.; Gulliver, F. D.; Forchheimer, L. L., and Alexander, R. C.: J. A. M. A. **121**:250, 1943.

187. Maumenee, A. E.; Hayes, G. S., and Hartman, T. L.: Am. J. Ophth. **28**:823, 1945.

188. Heyl, J. T.; Allen, H. F., and Cheever, F. S.: J. Immunol. **60**:37, 1948.

189. Ruchman, I.: Proc. Soc. Exper. Biol. & Med. **77**:120, 1951.

190. Cheever, F. S.: Proc. Soc. Exper. Biol. & Med. **77**:125, 1951.

191. Braley, A. E.: Personal communication to the authors.

192. Sezer, F. N.: A. M. A. Arch. Ophth **49**:293, 1953

identical to the one isolated in New York in 1942. A comparative study of the antigenic characteristics of the two agents is in progress. Bauer and Cooney [193] and Habel [194] have not been able to confirm the early results.

In view of the difficulty in confirming the first isolations of epidemic keratoconjunctivitis virus and the lack of clarity regarding the nature of the agent, it should be placed in Group II for additional investigation.

Embryonated Eggs.—Epidemic keratoconjunctivitis virus was carried through 12 passages on the chorioallantoic membrane of embryonated eggs (Calkins and Bond [195]), with an increase of virulence for the eggs but no change of viral characteristics with respect to mice. When the virus was established in eggs, embryo death occurred in three to four days. The agent was neutralized by human convalescent serums from the New York epidemic and by rabbit hyperimmune serum prepared against the New York virus.

EQUINE ABORTION VIRUS

Tissue Culture.—Randall and co-workers [196] were able to demonstrate the propagation of equine abortion virus in Maitland flask cultures with no difficulty. The fluid medium of the cultures consisted of 3 ml. of equal parts of Hanks' solution and human ascitic fluid. Spleen, lung, liver, chorioallantoic membrane, amnioallantoic membrane, and testis of 6 to 11 month horse fetuses were tested as tissue substrate. Since spleen and lung alone exhibited inclusion bodies in preliminary histological studies, these tissues were used exclusively in later passages. Ten to 15 pieces of 2 mm. tissue fragments were incubated in a 25 cc. Erlenmeyer flask for 24 hours before virus in the form of a high-speed centrifugate of infected foal lung was added. The tissue cultures were then incubated for five to seven days at 37 C. before passages were made with ground whole culture. The virus propagated through nine subcultures in spleen. This passage series represented a total dilution of $10^{16.8}$ of the original infectious tissue. Inclusion bodies which became more conspicuous upon passage were observed in spleen and lung incubated three to seven days with virus, but no inclusion bodies were demonstrated in plasma-clot preparations. Propagation of the equine abortion virus was also evidenced by the ability of the eighth subculture to induce abortion in 2 of 10 inoculated mares with characteristic lesions in their fetuses, and a definite rise in complement-fixing antibody was noted in all the animals. Tissue culture virus was further shown to be useful as complement-fixing antigen in dilutions representing a $10^{12.9}$ increase in virus through eight culture passages.

There is little doubt that the equine abortion virus has been propagated. However, the work presented is recent, and there has not yet been opportunity for confirmation. For this reason, the virus is placed, probably temporarily, in Group II.

Embryonated Eggs.—An attempt to grow the virus of equine abortion on the chorioallantoic membrane of the embryonated egg was unsuccessful (Goodpasture

193. Bauer, H., and Cooney, M.: Personal communication to the authors.

194. Habel, K.: Personal communication to the authors.

195. Calkins, H. E., and Bond, G. C.: Proc. Soc. Exper. Biol. & Med. **56**:46, 1944.

196. Randall, C. C.; Ryden, F. W.; Doll, E. R., and Schell, F. S.: Am. J. Path. **29**:139, 1953.

and Anderson [197]). However, when human amnion was grafted on the membrane and infected with emulsified liver of an aborted foal, nuclear inclusions, necrosis, and ulceration were observed in four experiments; human chorion yielded negative results.

EQUINE ENCEPHALOMYELITIS VIRUSES

Tissue Culture.—In 1933, Syverton, Cox, and Olitsky [198] reported propagation of an equine encephalomyelitis virus in tissue cultures. The virus employed in this and a subsequent study [199] was isolated by Meyer, Haring, and Howitt [200] in California and was later classified as the western strain. In a series of 53 culture generations with chick embryo and Tyrode's solution, the 49th subculture caused death in mice at a dilution of 10^{-5}, and the increase in potency was estimated quantitatively at 10^{61}. Cox [201] demonstrated that rapid multiplication of the virus occurred in 72 hours, and Zinsser and Schoenbach [202] noted that western equine encephalomyelitis virus in Maitland cultures multiplied during the period of active tissue metabolism, realizing its maximum titers in the fluid phase 12 to 24 hours after the cessation of cellular respiration.

Sanders and Molloy [54] studied the propagation of the viruses of eastern equine encephalomyelitis and western equine encephalomyelitis in Simms' solution-serum ultrafiltrate tissue cultures at room temperature and at 37 C. The highest titers of the western strain (10^{-5} to 10^{-7}) were obtained with clear supernatant fluid from the serum ultrafiltrate cultures maintained at room temperature. The virus grown in the cultures incubated at 37 C. yielded maximum titers of 10^{-5}, and, when Simms' solution alone was employed, the virus was less stable. The eastern strain grew equally well at room temperature and at 37 C. with titers of 10^{-7}. A formalized vaccine of low-protein content prepared from the virus propagated in tissue culture protected guinea pigs against 10,000 MLD of the virus.

Huang [89] devised a method for accurate in vitro titration which depended on the ability of the western strain to inhibit proliferation of plasma-patched, minced skeletal chick muscle. Titrations on the basis of pH changes, as with St. Louis encephalitis virus, were also shown to be feasible.[203]

The eastern strain can apparently be grown at will, and it is probable that investigators have used this agent for various studies without reporting propagation results. An example of the use of the eastern strain is seen in the investigation by Gey and Bang,[204] who compared the action of the virus in continuous culture on normal rat fibroblasts and tumor cell derivatives. Their results indicated that the malignant cell, as contrasted to its prototype, was highly susceptible, and, while no titers were noted, there appeared to be no difficulty in routine propagation of the virus.

197. Goodpasture, E. W., and Anderson, K.: Am. J. Path. **18**:563, 1942.
198. Syverton, J. T.; Cox, H. R., and Olitsky, P. K.: Science **78**:216, 1933.
199. Olitsky, P. K.; Cox, H. R., and Syverton, J. T.: J. Exper. Med. **59**:159, 1934.
200. Meyer, K. F.; Haring, C. M., and Howitt, B.: Science **74**:227, 1931.
201 Cox, H. R.: Proc. Soc. Exper. Biol. & Med. **33**:607, 1936.
202. Zinsser, H., and Schoenbach, E. B.: J. Exper. Med. **66**:207, 1937.
203. Huang, C. H.: Proc. Soc. Exper. Biol. & Med. **54**:160, 1943.
204. Gey, G. O., and Bang, F. B.: Cancer Res. **10**:219, 1950; Tr. New York Acad. Sc. **14**:15, 1951.

Embryonated Eggs.—The equine encephalomyelitis viruses have been cultivated at will on the chorioallantoic membrane and in the yolk sac of embryonated eggs (Higbie and Howitt [205]; Beard, Finkelstein, Sealy, and Wyckoff [206]; Bang [207]; Stimpert [208]). The viruses were uniformly lethal for young embryos, but neither specific lesions nor inclusion bodies were observed. Equine encephalomyelitis virus complement-fixing antigens were demonstrated in egg culture (Mohler [209]). Vaccines effective in both horses and humans were prepared from egg chorioallantois, and the allantoic cavity was suggested as the superior route of inoculation for this purpose (Beard, Finkelstein, Sealy, and Wyckoff [206]; Beard, Finkelstein, and Beard [210]).

FELINE PNEUMONITIS VIRUS

Embryonated Eggs.—The most satisfactory propagation of the feline pneumonitis virus occurred in the yolk sac (Hamre and Rake,[211] Baker,[212] Weiss [213]). The virus was also passed through embryonated eggs on the chorioallantoic membrane, but in two passages only 40% of the egg inoculated intra-allantoically gave evidence of infection (Weiss [213a]). The feline pneumonitis virus was found to propagate in the yolk sac of embryonated eggs chilled for 20 to 24 hours at 5 C. without changes in membrane appearance or in the virulence, antigenicity, or growth of the virus (Weiss [213b]). A toxin neutralized by homologous serum was demonstrated in heavily infected yolk sacs (Hamre and Rake [211]).

FOOT AND MOUTH DISEASE VIRUS

Tissue Culture.—Hecke [214] first cultivated the virus of foot and mouth disease using a preparation of embryonic guinea pig skin in plasma. He maintained the virus for 22 culture generations and 131 days. Since tissue necrosis was marked at 37 C. and there was a rapid destruction of the virus, he changed the incubation temperature to 30 C. and observed retardation of necrosis without loss of virus potency. At 37 C. he could maintain the cultures for only 49 days, whereas at 30 C. the virus could be cultivated for 81 days. This investigator had no difficulty in demonstrating a quantitative increase in virus as measured by infective dilutions of 1:10,000,000 in later cultures as compared to 1:10,000 in early cultures. He also grew the virus in adult guinea pig testes but found the rate slower, and continuous passages could not be maintained. Fetal lung cultures permitted a virus increase through 15 culture generations, and this study was repeated without difficulty by the same investigator.[215] In a later study [88] the virus of foot and mouth disease was found more closely combined with the cells than with the fluid com-

205. Higbie, E., and Howitt, B.: J. Bact. **29**:399, 1935.
206. Beard, J. W.; Finkelstein, H.; Sealy, W. C., and Wyckoff, R. W. G.: Science **87**:490, 1938.
207. Bang, F. B.: J. Exper. Med. **77**:337, 1943.
208. Stimpert, F. D.: Proc. Soc. Exper. Biol. & Med. **41**:483, 1939.
209. Mohler, W. M.: J. Am. Vet. M. A. **94**:39, 1939.
210. Beard, D. W.; Finkelstein, H., and Beard, J. W.: J. Immunol. **40**:497, 1941.
211. Hamre, D., and Rake, G.: J. Infect. Dis. **74**:206, 1944.
212. Baker, J. A.: J. Exper. Med. **79**:159, 1944.
213. Weiss, E.: (*a*) J. Infect. Dis. **84**:125, 1949; (*b*) ibid. **86**:27, 1950.
214. Hecke F.: Abstracted, Zentralbl. Bakt. (Abt. 1) **102**:283, 1931.
215. Hecke F.: Abstracted, Zentralbl. Bakt. (Abt. 1) **125**:321, 1932.

ponents of the cultures. This finding was confirmed by Striegler,[216] who cultivated the virus through 62 passages over a period of 316 days, with an estimated increase in potency of 10^{113}.

Maitland and Maitland [23] failed to obtain propagation of the virus in cultures of chick embryo in Tyrode's solution and chicken plasma. However, when the contents of a vesicle from an infected guinea pig were inoculated into cultures of the pads, lips, and tongue of a guinea pig, cultivation of the virus was observed through 17 subcultures, with the last subcultures producing infection. These were stored in the refrigerator for three months, and further subcultures were then made which showed an increase in virus after incubation in 50 cc. of medium in Roux bottles. The highest titers, 1:10,000, were observed after three or four days of incubation. Four guinea pigs which had recovered from infections induced by cultures were immune to normally infectious doses from guinea pig vesicles. It was also possible to cultivate the virus in embryonic guinea pig kidney and to a less degree in adult guinea pig kidney.

Frenkel,[22] in a report on the cultivation of foot and mouth disease virus in cultures of bovine tongue epithelium, noted his earlier (1933-1938) successes in propagating the virus in tissue cultures of embryonic bovine, porcine, and ovine skin. Thomas and co-workers [217] confirmed these observations with respect to the skin of bovine embryo in amniotic fluid. When the embryonic tissues were utilized, the virus did not grow with sufficient regularity or potency to be of practical use as a vaccine. Minced, deep epithelial lingual tissue of adult susceptible cattle, on the other hand, when cultured in Baker's solution, yielded virus titers of 10^{-5} to 10^{-6} in 24 hours.[22] Frenkel [218] adapted his method to mass virus culture (60-liter containers) and obtained satisfactory potencies. Subsequent refinements in technique, which included use of the entire lingual epithelium, as reported by Frenkel and Dunne,[219] produced virus titers as high as 1:920,000. Frenkel and Frederickes [220] also reported the propagation of foot and mouth virus in epithelial tissue of bovine rumen.

Mace and co-workers,[221] working in Mexico, were unable to cultivate the MP VI strain of the Valee A type foot and mouth disease virus using the methods of Frenkel and Dunne [219] with minor modifications of the fluid medium. However, when Roux bottles with bottom surface areas of 35 sq. in. were filled to a depth of ¼ in. with 18 gm. of lingual epithelium suspended in modified Baker's solution and 20% serum ultrafiltrate, 40 serial passages were completed. The infectious titers varied through the series from 10^{-2} to $10^{-6.5}$ but were for the most part within a 10^{-5} to $10^{-6.5}$ range. While the addition of vitamins and amino acids to the nutrient medium did not affect virus yields, the removal of the serum ultrafiltrate did decrease the yields. No change in antigenic character could be demonstrated, and preliminary trials with an aluminum hydroxide colloid vaccine were satisfactory.

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216. Striegler, E.: Zentralbl. Bakt. (Abt. 1) **128**:332, 1933.

217. Thomas, J. A.; Thiery, J. P.; Salomon, L.; Salomon, L., and Thiery, J. P.: Compt. rend. Acad. sc. **233**:506, 1951.

218. Frenkel, H. S.: Am. J. Vet. Res. **12**:187, 1951.

219. Frenkel, H. S., and Dunne, H. W.: Am. J. Vet. Res. **13**:21, 1952.

220. Frenkel, H. S., and Frederiks, H. H. J.: Nature, London **164**:235, 1949

221. Mace, D. L.; Dunne, H. W.; Eichhorn, A., and Camargo, F.: J. Infect. Dis. **88**:212, 1951.

According to Frenkel [222] the histopathology of the vesicle formation in foot and mouth disease virus cultures is very similar to that seen in the diseased animal. No inclusion bodies were observed.

Embryonated Eggs.—Unsuccessful attempts to culture the virus of foot and mouth disease have been reported (Galloway and Elford,[223] Richter [224]). In 1948 a positive report by Traub [225] appeared which may have an important bearing on future studies. It is discussed in some detail. Mixtures of 10 different virus strains of foot and mouth disease virus were inoculated in embryonated eggs. The eggs were incubated at 37 C. for 10 days in the first passage and for 9 days in succeeding passages. Attempts to carry the virus in serial passage from egg to egg were unsuccessful. For that reason the virus was maintained by alternate egg and guinea pig passage, and after 4 alternate passages the virus was maintained in eggs for 30 consecutive passages. The best results were obtained by inoculation of the chorioallantoic membrane, followed by an incubation period of 40 to 48 hours. In one of the 10 direct egg passages, embryo extract was used as subinoculum. Infection of the embryo was noted between 24 and 48 hours after inoculation. There was more virus in 11 to 13-day-old embryos than 7 to 9-day embryos. The specific character of the 10 virus strains was determined, and some immunologic alteration was noted, apparently resulting from continued egg passage. There was attenuation of the highly pathogenic strain "A" for guinea pigs in the course of continuous egg passages. No specific membrane or embryo changes due to foot and mouth disease infection were noted.

FOWL LEUCOSIS VIRUS

Tissue Culture.—Furth and Stubbs [226]; Vern, Oberling, and Guerin,[227] and Furth and Breedis [228] have all reported maintenance of leukemic cells in tissue culture preparations. As a result of their experiments with strains of leucosis sarcomaleucosis, osteochondrosarcoma, and lymphomatosis, Furth and Breedis concluded that "oncogenic viruses multiply in vitro only in the presence of cells on which they confer neoplastic properties."

Doljanski and Pikovski,[229] in direct contradiction to this statement, were able to propagate the virus of fowl leucosis on fibroblasts in Carrel flasks. Colonies of fibroblasts derived from normal chick heart were bathed for one hour in a cell-free (Berkefeld N) filtrate of spleen and heart from leucotic birds. The cultures were then washed with normal chicken plasma and fed with this fluid. Control flasks without tissue were prepared, and the virus in these preparations was not demonstrable after two weeks. In the presence of fibroblasts, infectivity of the ultrafiltrated wash fluids, as well as of the culture, was evidenced after 86 days and four culture passages.

222. Frenkel, H. S.: Am. J. Vet. Res. **10**:142, 1949.
223. Galloway, I. A., and Elford, W. J.: Brit. J. Exper. Path. **16**:588, 1935.
224. Richter, H. A.: Zentralbl. Bakt. (Abt. 1) **143**:273, 1939.
225. Traub, E., and Schneider, B.: Ztschr. Naturforsch. **3b**:178, 1948.
226. Furth, J., and Stubbs, E. L.: Proc. Soc. Exper. Biol. & Med. **32**:381, 1934.
227. Verne, J.; Oberling, C., and Guerin, M.: Compt. rend. Soc. biol. **121**:403, 1936.
228. Furth, J., and Breedis, C.: Arch. Path. **24**:281, 1937.
229. Doljanski, L., and Pikovski, M.: Nature, London **146**:302, 1940.

FOWL PLAGUE VIRUS

Tissue Cultures.—In 1931, Hallauer [36] reported cultivation of the virus of fowl plague for nine subcultures over a period of 88 days. Increased virulence was demonstrated when embryonic chick brain was used in Carrel-flask preparations. The highest concentration of virus occurred during the fourth day of cultivation and was followed by a rapid loss of potency. However, when the Maitland type of culture was used, virus growth was more gradual, and fall in titer was slower.[230]

Using Erlenmeyer-flask preparations of chick embryonic tissue in Drew's solution, Plotz [231] was successful in passing the virus through 15 culture generations with dilution of the original inoculum to 248^{-9}.

Plotz and Ephrussi [88a] demonstrated the ability of normal tissue to proliferate if transferred after seven days from the fluid medium to hanging drop preparations. However, if tissue from infected flasks was taken, proliferation rarely occurred because of cellular damage wrought by the virus. The same authors [88b] also showed that the virus of fowl plague was able to propagate in the presence of cells which were viable but not in a state of proliferation. Under such circumstances 10th passage virus in a dilution of 1 : 1,000,000 was able to infect and kill a chicken.

By using a strain of virus cultivated on embryonic chick liver cells, Hallauer [232] was able to immunize chickens in two weeks against massive doses of virus. The liver tissue appeared to have the faculty of lowering virulence without reducing antigenicity. The inactivation of the virus in these cultures depended on the amount of inoculum, the degree of cellular proliferation, and the duration of cultivation. In vitro experiments with immune serum yielded results similar to those reported by Rivers, Haagen, and Muckenfuss with vaccinia.[233] When immune serum was added to the explanted tissue before the addition of virus, neutralization of the virus occurred; when it was added after the cells had been in contact with the virus, little or no neutralizing effect was observed.

Plotz [234] reported that the virus of fowl plague in tissue cultures increased 50 times in 24 hours and 100 times in 48 hours. After 28 culture transfers a dilution of 1 : 1,000,000 was fatal to chickens. He found also that too much or too little tissue in the cultures inhibited the formation of virus. In another series of experiments [234b] he noted that embryonic canary was as favorable a medium for this virus as embryonic chick.

By 1934, Plotz [235] had cultivated a strain of virus for two and one-half years, making subcultures every three days. And in 1937 he reported propagation of the strain for five years through 250 culture generations.[236]

Embryonated Eggs.—The virus of fowl plague has been cultured on the chorioallantoic membrane and in the allantoic cavity (Burnet and Ferry,[237] Kimura and

230. Hallauer, C.: Ztschr. Hyg. **115**:616, 1933.
231. Plotz, H.: Compt. rend. Soc. biol. **110**:163, 1932.
232. Hallauer, C.: Ztschr. Hyg. **116**:456, 1934.
233. Rivers, T. M.; Haagen, E., and Muckenfuss, R. S.: J. Exper. Med. **50**:673, 1929.
234. Plotz, H.: (*a*) Compt. rend. Soc. biol. **113**:570, 1933; (*b*) Compt. rend. Acad sc. **196**:1545, 1933; (*c*) ibid. **197**:536, 1933; (*d*) Compt. rend. Soc. biol. **113**:1495, 1933.
235. Plotz, H.: Compt. rend. Soc. biol. **116**:956, 1934.
236. Plotz, H.: Compt. rend. Soc. biol. **125**:602, 1937.
237. Burnet, F. M., and Ferry, J. D.: Brit. J. Exper. Path. **15**:56, 1934.

Masunaga,[238] Haber,[239] Lush [240]). Inoculation by the former route caused the death of embryos without specific changes in the membrane (Burnet and Ferry [237]). When 9-day-old embryos were used, survival time was three to four days, and liver lesions and inclusion bodies were observed 24 hours after infection (Haber [239]). A 1 : 1,000 dilution of infected allantoic fluid inoculated intra-allantoically killed embryos in a little more than 24 hours (Lush [240]). Utilizing intra-allantoic inoculation, red blood cell agglutinins were demonstrated; these serve to differentiate the virus of the fowl plague from the virus of Newcastle disease. The growth of the virus was followed with the phase and electron microscopes (Flewett and Challice [241]).

FOWL POX VIRUS

Tissue Culture.—In 1928, Findlay [25] and Loewenthal [242] succeeded in cultivating the virus of fowl pox for a small number of generations. Flask cultures containing embryonic chick brain and skin were used by Findlay, who was able to demonstrate by the fourth generation a level of potency 20 times that of the original virus. However, the fourth subculture showed a decrease in titer from that of the second and third subcultures. This work was later repeated by Bierbaum and Gaede,[243] who maintained the virus for 12 culture passages.

More recently, Bang, Levy, and Gey [244] have grown the virus of fowl pox in continuous roller tube cultures of chick embryo fibroblasts. The virus survived under these conditions but multiplied only to very low titers (at times the virus was not demonstrable in undiluted fluid by egg inoculation). Neither cellular destruction nor intracellular inclusion bodies were evident in these cultures. Chick corneal epithelium also supported virus propagation, but with this tissue marked destruction of the epithelial cells was observed.

The data from the later work with this virus do not seem to require revision of the statement made here in 1939 that the increase in potency of fowl pox virus in tissue cultures is small and unpredictable.

Embryonated Eggs.—Fowl pox virus was shown to grow on the chorioallantoic membrane of the embryonated egg (Woodruff and Goodpasture [245] Burnet [246]). Skin grafts on the membrane also supported virus propagation (Goodpasture and Anderson [247]). The virus appeared in the chorioallantoic membrane in greater quantities than in the yolk sac (Haig [248]), and the lesions on the membrane varied considerably with the concentration of the inoculum and the number of previous egg passages (Burnet [246]).

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238. Kimura, R., and Masunaga, N.: Zentralbl. Bakt. (Abt. 1) **143**:165, 1939.
239. Haber, P.: Compt. rend. Soc. biol. **126**:885, 1937.
240. Lush, D.: J. Comp. Path. & Therap. **53**:157, 1943.
241. Flewett, T. H., and Challice, C. E.: J. Gen. Microbiol. **5**:279, 1951.
242. Loewenthal, H.: Klin. Wchnschr. **7**:349, 1928.
243. Bierbaum, K., and Gaede, H.: Arch. wissensch. u. prakt. Thierh. **69**:441, 1935.
244. Bang, F. B.; Levy, E., and Gey, G. O.: J. Immunol. **66**:329, 1951.
245. Woodruff, A. M., and Goodpasture, E. W.: Am. J. Path. **7**:209, 1931.
246. Burnet, F. M.: Special Report Series No. 220, Medical Research Council, London, Her Majesty's Stationery Office, 1936.
247. Goodpasture, E. W., and Anderson, K.: Arch. Path. **30**:212, 1940.
248. Haig, D. A.: Onderstepoort, J. Vet. Sc. **25**:17, 1951.

The close correlation between the number of membrane lesions and virus potency made it possible to carry out titrations by inoculation of the chorioallantois (Burnet and Lush,[249] Haig [248]).

The appearance of the chorioallantoic membrane infected with fowl pox virus was carefully studied with the electron microscope (Morgan and Wyckoff [250]; Bang, Levy, and Gey [244]).

Kikuth's canary pox virus was also cultivated on the chorioallantoic membrane and found to be serologically similar to the fowl pox virus (Burnet and Barnard,[251] Burnet and Lush [252]).

HERPES SIMPLEX VIRUS

Tissue Culture.—Propagation of the herpes simplex virus through 10 culture generations was reported by Parker and Nye [253] in 1925. These authors used an emulsion of infected rabbit brain or testicle to inoculate hanging drop preparations of rabbit testicular tissue and plasma. The virus was not demonstrable after the 10th subculture. Gildemeister, Haagen, and Scheele [254]; Andrewes [255]; Saddington,[256] and Haagen [257] all confirmed the ability of the herpes virus to propagate in the presence of rabbit testicle. Haagen [258] also employed chick embryo tissue, and rabbit cornea was used by Mitamura and co-workers.[259] Of these investigators, only Haagen [257] was able to demonstrate the presence of virus for more than 25 subcultures. Gildemeister and co-workers [254] noted attenuation of the virus and loss of neurotropism concomitant with the absence of inclusion bodies. Andrewes,[255] on the other hand, observed inclusion bodies in tissue culture and retention of neurotropic properties. In L-strain cells Scherer [260] reported the presence of typical intranuclear inclusion bodies.

Cheever [261] obtained virus multiplication on agar slant cultures of chick embryo tissue. He observed that, although tissue cultures as old as 17 days permitted the survival of herpes simplex virus for at least 96 hours, 4-day or older cultures did not allow indefinite serial passage of the virus as did fresh tissue.[261b]

Stulberg and Schapira [262] found virus multiplication to occur in chick embryo lung-derived fibroblasts cultured in plasma drop preparation but not in similar cultures of heart muscle fibroblasts, and in neither case was cell destruction evident.

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249. Burnet, F. M., and Lush, D.: Brit. J. Exper. Path. **17**:302, 1936.
250. Morgan, C., and Wyckoff, R. W. G.: J. Immunol. **65**:285, 1950.
251. Burnet, F. M., and Barnard, J. E.: J. Path. & Bact. **37**:107, 1933.
252. Burnet, F. M., and Lush, D.: J. Path. & Bact. **43**:105, 1936. Footnote 249.
253. Parker, F., and Nye, R. N.: Am. J. Path. **1**:337, 1925.
254. Gildemeister, E.; Haagen, E., and Scheele, L.: Zentralbl. Bakt. (Abt. 1) **114**:309, 1929.
255. Andrewes, C. H.: J. Path. & Bact. **33**:301, 1930.
256. Saddington, R. S.: Proc. Soc. Exper. Biol. & Med. **29**:1012, 1932.
257. Haagen, E.: Zentralbl. Bakt. (Abt. 1) **120**:304, 1931.
258. Haagen, E.: Zentralbl. Bakt. (Abt. 1) **129**:251, 1933.
259. Mitamura, T.; Kitaoka, M.; Watanabe, S., and Ohkubo, K.: Tr. Soc. path. jap. **25**:122, 1935.
260. Scherer, W. F.: Am. J. Path. **29**:113, 1953.
261. (*a*) Cheever, F. S.: Proc. Soc. Exper. Biol. & Med. **42**:113, 1939. (*b*) Cheever, F. S., and Willmert, G. P.: ibid. **51**:35, 1942.
262. Stulberg, C. S., and Schapira, R.: J. Immunol. **70**:51, 1953.

Both lung and heart muscle fibroblasts supported the propagation of herpes simplex virus in cellophane substrate cultures, and in this instance virus growth was accompanied by destruction of cells.

Scherer [260] passed herpes simplex virus through 10 cultures of L-strain fibroblasts grown on glass. This investigator found maximum virus multiplication in a culture preparation containing Hanks' solution, horse serum, and embryo extract ultrafiltrate (the nutrient medium providing the highest functional state of cells). When serum ultrafiltrate was substituted as the fluid phase, the virus growth was significantly less than that in the Hanks' solution-horse serum-embryo extract ultrafiltrate medium. Herpes simplex virus multiplied minimally, if at all, in maintenance tissue cultures, i. e., cells were maintained but did not proliferate in the presence of an amino-acid-augmented salt solution.

Embryonated Eggs.—Herpes simplex virus grew in the embryonated egg when inoculated on the chorioallantoic membrane,[263] in the amnion,[264] in the embryo brain,[264] in the yolk sac,[265] and in and under the skin of the embryo [264] (Saddington [256]; Dawson [263e]; Burnet, Lush, and Jackson [263d]; Burnet and Lush [263b,c]; Shaffer and Enders [263f]; Nagler [265]; Anderson [264]; Balderidge and Blank [263a]). The effects on the egg varied with the mode of infection, but in all cases there was a high embryo mortality.

With passage, the virus became adapted to the egg milieu as measured by a decrease in virulence for mice and rabbits and an increase in egg pathogenicity (Anderson,[264] Beveridge and Burnet,[5] Shaffer and Enders [263f]).

Some workers have reported specific nuclear changes and even typical inclusion bodies, but a careful electron microscope study of the infected chorioallantoic membrane did not substantiate such findings (Bang [266]). Grossly proliferative and necrotic lesions occurred on the chorioallantoic membrane; these increased in size with egg passage.

HERPES ZOSTER VIRUS

Tissue Cultures.—Glaubersohn and Barg,[267] in 1934, inoculated the contents of herpetic vesicles into hanging drops made up of chick embryo heart tissue, rabbit plasma, and Tyrode's solution. After incubation for four days at 37 C. and two days at room temperature, the culture material was inoculated into the arm of an infant. The results were one positive reaction, i. e., vesicle formation, and one negative reaction. No subcultures and no estimate of increase in potency were made. As Glaubersohn and Barg suggested, the presence of virus in the tissue cultures may have been due to survival of the original inoculum.

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263. (*a*) Balderidge, G. D., and Blank, H.: Proc. Soc. Exper. Biol. & Med. **72**:560, 1949. (*b*) Burnet, F. M., and Lush, D.: J. Path. & Bact. **49**:241, 1939; (*c*) Lancet **1**:629, 1939. (*d*) Burnet, F. M.; Lush, D., and Jackson, A. V.: Australian J. Exper. Biol. & M. Sc. **17**:35, 1939. (*e*) Dawson, J. R.: Am. J. Path. **9**:1, 1933. (*f*) Shaffer, M. F., and Enders, J. F.: J. Immunol. **37**:383, 1939. (*g*) Saddington.[256]

264. Anderson, K.: Am. J. Path. **16**:137, 1940.

265. Nagler, F. P. O.: Unpublished data, in Beveridge, W. I. B., and Burnet, F. M.: Special Report Series No. 256, Medical Research Council, London, Her Majesty's Stationery Office, 1946.

266. Bang, F. B.: Bull. Johns Hopkins Hosp. **87**:511, 1950.

267. Glaubersohn, S. A., and Barg, G. S.: Acta med. scandinav. **82**:579, 1934.

Embryonated Eggs.—Success in cultivating herpes zoster virus has been erratic. In 1936 one worker (de Castro Teixeira [268]) reported three passages on the chorioallantoic membrane of 12 to 13-day eggs. This work was not confirmed, but later evidence was found of virus proliferation in one of four attempts to grow herpes zoster virus on grafted human skin (Goodpasture and Anderson [269]). Another laboratory demonstrated intranuclear inclusion bodies in membrane-grafted human prepuce (Blank, Coriell, and Scott [270]). In neither case was any infection of membranal cells observed.

HOG CHOLERA VIRUS

Tissue Culture.—The virus of hog cholera propagated temporarily in tissue culture, as noted by Hecke [271] in 1932. Explants of choroid plexus, lymph nodes, bone marrow, spleen, and kidney were cultured in hanging drop and flask preparations. Hanging drop cultures of choroid plexus, plasma, and spleen extracts were infective for 15 culture passages. With bone marrow and lymph node tissue suspended in Drew's solution, the virus maintained virulence for 10 to 20 passages, respectively. Lymph node cultures retained infectivity after a dilution of 10^{30} of the original material, and infected spleen cultures were pathogenic after a dilution of 10^{20} in the 14 subcultures.

TenBroeck [272] was able to demonstrate propagation of hog cholera virus in flask cultures of minced swine testicle, as well as in agar slant preparations of the same tissue. Fourteen passages were carried out in the flask preparations, while virus in the tissue agar cultures was maintained for 13 transfers without any loss of virulence; in fact, it was suggested that the cultured virus was more potent than that found in infectious serum. Boynton and co-workers [273] cultured the hog cholera virus for 89 generations using bone marrow suspended in serum ultrafiltrate. After 52 subcultures a loss of pathogenicity for hogs was observed. Only a temperature rise was observed when the last culture passage virus was inoculated into pigs; no symptoms appeared. Boynton [274] noted that when an ultrafiltrate of immune pig serum was used in the fluid phase virulence of the culture was not affected. Preliminary trials with a culture vaccine were encouraging.

Embryonated Eggs.—In one series of experiments, no growth of hog cholera virus was observed after inoculation on the chorioallantoic membrane or in the veins of the embryo (TenBroeck [272]). Cultures of minced swine testicle implanted on the chorioallantoic membrane did, however, support virus propagation.

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268. de Castro Teixeira, J.: Compt. rend. Soc. biol. **121**:781, 1936.

269. Goodpasture, E. W., and Anderson, K.: Am. J. Path. **20**:447, 1944.

270. Blank, H.; Coriell, L. L., and Scott, T. F. M.: Proc. Soc. Exper. Biol. & Med. **69**:341, 1948.

271. Hecke, F.: Zentralbl. Bakt. (Abt. 1) **126**:517, 1932.

272. TenBroeck, C.: J. Exper. Med. **74**:427, 1941.

273. Boynton, W. H.; Takahashi, W. N.; Woods, G. M., and Walker, W. W.: Vet. Med. **43**:403, 1948.

274. Boynton, W. H.: Vet. Med. **41**:346, 1946.

INCLUSION BLENNORRHEA VIRUS

Tissue Culture.—In a thorough study of the cultivation of the virus of inclusion blennorrhea, Braley [275] found it impossible to obtain propagation of the virus in tissue culture preparations. The virus did not multiply in Simms' solution-ultrafiltrate flask cultures of infected conjunctival epithelium scrapings, and no virus could be recovered from Carrel-type plasma cultures of normal conjunctival tissues inoculated with the virus. When infected tissue was explanted according to Thygeson's [276] method for trachomatous tissue, cellular proliferation occurred.

Embryonated Eggs.—An attempt to demonstrate propagation in the embryonated egg when the virus of inclusion blennorrhea was inoculated onto the chorioallantoic membrane was entirely negative (Braley [275]).

VIRUS OF INFECTIOUS BRONCHITIS OF CHICKS

Embryonated Eggs.—Beaudette [277] passed through chorioallantoic preparations a virus that produced fatal bronchitis in chickens which had been vaccinated against infectious laryngotracheitis. Berkefeld filtrates were used as the inoculums. Fourteen egg passages were reported, with an apparent increase in virulence. From the seventh generation on, the mortality of the embryos increased, and the period of incubation decreased. The chorioallantoic membranes did not show lesions comparable to those of infectious laryngotracheitis or of the poxes. No histologic studies were done.

INFECTIOUS HEPATITIS VIRUS

Tissue Cultures.—In view of the consistently negative or, at best, questionable results of all previous attempts to obtain propagation of the infectious hepatitis virus in nonhuman hosts and in tissue culture (Colbert [278]), the apparent success of Henle and co-workers [40] with embryonated eggs and suspended cell cultures is of special importance.

In a series of comprehensive experiments, Henle and his co-workers were able to demonstrate multiplication of two strains (Akiba and NL) of infectious hepatitis virus in cultures of minced 10 to 13-day chick embryo (eyes, beak, and claws removed) suspended in Simms' solution-serum ultrafiltrate. Virus propagation was also observed in roller tube cultures of 1 to 4-day rabbit liver modified to suppress fibroblastic proliferation. With the Akiba strain, six passages were carried out in each culture milieu, and sonically homogenized suspended cell cultures, as well as nutrient fluid from the roller tube culture, produced clinically apparent mild hepatitis in 14 of 18 individuals infected orally and subcutaneously. The total dilution of the original inoculum was calculated to be $10^{-7.7}$. Material from the final cultures was stored for 18 months at — 20 C. before four further passages were attempted in chick cell suspensions. The 10th culture generation constituted a 10^{-13} accumulative dilution of the original virus, and 4 ml. of sonically homogenized 10th generation culture administered orally produced clinical hepatitis in two of four volunteers.

With the NL strain, after 10 consecutive weekly passages, the homogenized cultures proved infectious for three of five individuals who exhibited signs of clinical hepatitis.

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275. Braley, A. E.: Arch. Ophth. **41**:151, 1949.
276. Thygeson, P.: Arch. Ophth. **21**:229, 1939.
277. Beaudette, F. R.: Poultry Sc. **16**:103, 1937.
278. Colbert, J. W.: Yale J. Biol. & Med. **21**:335, 1949.

The specificity of the tissue culture agent was tested in seven volunteers previously exposed to the virus. Of the six who had shown clinical and laboratory illness in the first tests, all were resistant to the challenge of natural virus. The remaining volunteer, who had shown no signs of illness after his first exposure, contracted hepatitis on the second challenge.

A skin test (Henle and co-workers [279]) with pooled amniotic fluid from the fifth to seventh egg passage following 10 tissue culture passages appeared to corroborate the identity of the agent. Still, Henle and his associates are reluctant to claim final identification until a specific serologic test is developed.

Embryonated Eggs.—When Colbert tabulated the results of findings of investigations directed toward culturing an agent of infectious hepatitis in the embryonated egg, all were classified as negative or highly questionable (Colbert [278]). However, in 1950 the growth of the virus in the amnion of fertile eggs was reported, using a 10th tissue culture generation virus as the original inoculum (Henle and co-workers [40]). The third egg passage was tested in human volunteers and produced clinical evidence of hepatitis. Ultraviolet-irradiated amniotic fluids were employed with apparently considerable specificity as a skin test antigen (Henle and co-workers [279]).

INFECTIOUS LARYNGOTRACHEITIS VIRUS

Embryonated Eggs.—The virus of infectious laryngotracheitis was cultivated on the chorioallantoic membrane and in the amniotic cavity (Burnet,[280] Brandly,[281] Burnet and Foley [282]). When inoculated into the amniotic sac of embryonated eggs, a turbidity of the fluid without appreciable decrease in volume resulted.[282] In general, the chorioallantoic membrane lesions are similar to those of vaccinia (Beveridge and Burnet [5]). According to Cox,[127] a protective vaccine for fowls has been prepared from whole eggs minus yolk sac infected via the allantoic sac.

INFECTIOUS MONONUCLEOSIS

Tissue Culture.—Van den Berghe and Liessens [283] have claimed transmission of infectious mononucleosis to Macacus rhesus monkeys by injecting subcutaneously the blood from an infected infant. According to these authors, they produced a syndrome in the monkeys characterized by leucocytosis or a suggestive leucopenia and by increased heterophile agglutinin titer (Paul and Bunnell test). They also stated that it was possible to maintain the disease by monkey passage following E K Seitz filtration of the blood.

Van den Berghe, Liessens, and Kovacs [284] employed a suspension of minced embryonic chick in 10% human serum-Tyrode's solution incubated at 37 C. Subcultures were made every three to four days. These workers claim multiplication of the virus in such tissue cultures as demonstrated by the production of a "disease"

279. Henle, G.; Drake, M. E.; Henle, W., and Stokes, J.: Proc. Soc. Exper. Biol. & Med. **73**:603, 1950.

280 Burnet, F. M.: Brit. J. Exper. Path. **15**:52, 1934; J. Exper. Med. **63**:685, 1936.

281. Brandly, C. A.: J. Infect. Dis. **57**:201, 1935.

282. Burnet, F. M., and Foley, M.: Australian J. Exper. Biol. & M. Sc. **19**:235, 1941.

283. van den Berghe, L., and Liessens, P.: Compt. rend. Soc. biol. **130**:279, 1939.

284. van den Berghe, L.; Liessens, P., and Kovacs, L.: Compt. rend. Soc. biol. **131**:156, 1939.

in three monkeys with 1st, 5th, and 10th culture passage material. In the final test-animal inoculated with the 10th culture passage, monocytes increased from 0.5% prior to infection to 31% after infection, and there was a drop in leucocytes from 34,000 to 9,000 after the inoculation of culture. The Paul and Bunnell agglutinin titer rose from 1 : 28 to 1 : 224, as measured macroscopically.

No control procedures have been described in the single report of positive findings. In view of Julianelle's [285] results with normal chick tissue and normal human blood injected into monkeys and rabbits, which stimulated leucocytosis and heterophile agglutinins, control studies are of vital importance in evaluating monkey reaction to injected material.

Embryonated Eggs.—One investigator reported positive results in four of eight attempts to pass a filterable agent from nasal washings and whole blood of humans suffering from a disease diagnosed as infectious mononucleosis (Nettleship [286]). Evidence for virus multiplication consisted of "small, pearl-gray reactions with a spreading cloudiness" on the chorioallantoic membrane. The agent was not maintained, and no human experiments were described.

INFLUENZA VIRUS

Tissue Culture.—Francis and Magill [287] cultivated the virus of influenza in vitro by inoculating lung tissue from infected mice into flasks of Tyrode's solution and chick embryo. They maintained virulence of the agent for 20 subcultures and were also successful in propagating swine and human influenza viruses, obtaining titers of 10^{-3} and 10^{-4} in ferrets and mice. Smith [288] reported propagation of an egg-adapted strain in a similar medium, and the neurotropic variant of the WS strain was grown in Maitland type cultures by Andrewes.[289]

Tissue cultures other than the suspended cell type have also been employed for cultivation of the influenza virus. Pearson and Enders [290] used roller tubes. Bernkopf [34] obtained unusually high hemagglutinin titers with deembryonated egg preparations. Intact chorioallantoic membranes in Warburg flasks were found by Ackermann [291] to be highly satisfactory for virus multiplication. Cushing and Morgan [81] were able to propagate the influenza virus in the presence of minced chick embryo tissue overlaid with a perforated cellophane disc.

In contrast to the earlier work, Lennette and Koprowski [292] found it necessary to increase the quantity of tissue from 0.1 gm. to at least 0.2 gm. in 50 ml. Erlenmeyer flasks before a tissue culture strain of PR8 could be established. The salutary effect of increased tissue upon influenza virus propagation was also demonstrated by Daniels, Eaton, and Perry,[293] who further noted an increase in hemagglutinin titer when the cultures were agitated, a practice employed by Ackermann.[291]

285. Julianelle, L. A.; Bierbaum, O. S., and Moore, C. V.: Ann. Int. Med. **20**:281, 1944.
286. Nettleship, A.: Proc. Soc. Exper. Biol. & Med. **49**:116, 1942.
287. Francis, T., and Magill, T. P.: Science **82**:353, 1935.
288. Smith, W.: Brit. J. Exper. Path. **16**:508, 1935.
289. Andrewes, C. H.: Brit. J. Exper. Path. **23**:214, 1942.
290. Pearson, H. E., and Enders, J. F.: Proc. Soc. Exper. Biol. & Med. **48**:140, 1941.
291. Ackermann, W. W.: J. Biol. Chem. **189**:421, 1951.
292. Lennette, E. H., and Koprowski, H.: J. Exper. Med. **83**:195, 1946.
293. Daniels, J. R.; Eaton, M. D., and Perry, M. E.: J. Immunol. **69**:321, 1952.

Pearson and Enders [290] investigated the tissue selectivity of influenza A virus in roller tube cultures. It was found that the virus grew well in lungs, intestine, and skin tissue of the chick embryo but poorly in brain, skeletal muscle, and heart. In the suspended cell cultures of Weller and Enders,[82] embryonic brain tissue gave satisfactory virus propagation. Stulberg and Schapira [294] demonstrated that an egg-adapted PRB strain failed to propagate in fibroblast cells derived by passage from chick embryo lung, heart, skeletal muscle, or corneal tissue grown in roller tube and Earle's cellophane cultures. Agreeing with Pearson and Enders, these workers found that the virus propagated (without cytopathogenic effects) in primary cultures of lung and cornea but not in similar cultures of heart and skeletal muscle.

In view of the variety of methods for assaying the influenza virus content of cultures, i. e., egg infectivity, mouse infectivity, and hemagglutinin titer, and the number of virus strains studied, it is difficult to compare different cultural methods. The one-hundred-thousand-fold increase in egg infectivity in 27 hours reported by Ackermann [291] and the hemagglutinin titers of Bernkopf [34] and Daniels, Eaton, and Perry [293] suggest the superiority of their respective techniques.

There appears to be general agreement among the investigators [295] that the influenza virus multiplies with great rapidity in tissue culture, maximum titers occurring after one [34] to five days [31] of incubation at 37 C. Attention is directed to the recent contribution of Ackermann and Kurtz,[296] who propagated the virus of influenza in a new host system (the ascites fluid produced in the presence of Ehrlich mouse carcinoma), which may be considered more of an in vivo than an in vitro technique.

Embryonated Eggs.—The influenza virus has probably been the most thoroughly studied virus in embryonated eggs. The literature on this subject is voluminous and only a bare outline is given.

The virus of influenza has been successfully cultivated in embryonated eggs after the following inoculations: chorioallantoic membrane (Smith [288]), amniotic sac (Burnet, Beveridge, Bull, and Clarke [297]; Hirst [298]; Burnet, Beveridge, and Bull [299]), yolk sac (Nigg, Crowley, and Wilson [300]; Burnet, Beveridge, and Bull [299]), and allantoic sac (Burnet [301]; Rickard, Thigpen, and Crowley [302]; Eaton, Corey, Van Herick, and Meiklejohn [303]; Henle and Chambers [304]; Henle and Henle [305]; Miller [306]).

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294. Stulberg, C. S., and Schapira, R.: J. Immunol. **70**:51, 1953.

295. Packalen, T.: Acta path. et microbiol. scandinav. **23**:512, 1946. Smith.[288] Ackermann.[291] Weller and Enders.[82] Cushing and Morgan.[31]

296. Ackermann, W. W., and Kurtz, H.: Proc. Soc. Exper. Biol. & Med. **81**:421, 1952.

297. Burnet, F. M.; Beveridge, W. I. B.; Bull, D. R., and Clark, E.: M. J. Australia **2**:371, 1942.

298. Hirst, G. K.: J. Immunol. **45**:293, 1942; Proc. Soc. Exper. Biol. & Med. **58**:155, 1945.

299. Burnet, F. M.; Beveridge, W. I. B., and Bull, D. R.: Australian J. Exper. Biol. & M. Sc. **22**:9, 1944.

300. Nigg, C.; Crowley, J. H., and Wilson, D. E.: Science **91**:603, 1940.

301. Burnet, F. M.: Australian J. Exper. Biol. & M. Sc. **19**:291, 1941.

302. Rickard, E. R.; Thigpen, M., and Crowley, J. H.: J. Immunol. **49**:263, 1944.

303. Eaton, M. D.; Corey, M.; Van Herick, W., and Meiklejohn, G.: Proc. Soc. Exper. Biol. & Med. **58**:6, 1945.

304. Henle, W., and Chambers, L. A.: Proc. Soc. Exper. Biol. & Med. **46**:713, 1941.

305. Henle, W., and Henle, G.: Science **98**:87, 1943.

306. Miller, G. L.: J. Exper. Med. **79**:173, 1944

Focal lesions on the chorioallantoic membrane presented themselves, and a fatal hemorrhagic infection resulted after membranal inoculation. Amniotic sac inoculation killed the embryos, produced turbidity in the amniotic fluid, and diminished the volume of this fluid. Following yolk sac inoculation, the embryos died in four to eight days, and virus injected into the allantoic cavity acted similarly. Complement-fixing antigen was demonstrated in the yolk sac,[300] and hemagglutinins were demonstrated in the allantoic fluid (Hirst,[308] McClelland and Hare [309]). Extensive investigations of virus propagation (Henle,[75] Henle and Henle,[76] Hoyle,[74] Hoyle,[80] Cairns and Edney,[310] von Magnus [311]), interference (Ziegler and Horsfall [312]; Ziegler, Lavin, and Horsfall [313]; Henle and Henle [314]; Isaacs and Edney [315]), and alterations in viral characteristics (Burnet and Bull,[316] Burnet and Stone,[317] Hirst,[318] Archetti and Horsfall,[319] Archetti,[320] Sugg [321]) have been undertaken in the influenza virus-embryonated egg system.

Excellent vaccines have been prepared from egg-propagated virus (Francis and Salk,[322] Stanley,[323] Friedewald [324]).

LOUPING ILL VIRUS

Tissue Cultures.—Rivers and Ward [325] were successful in passing the virus of louping ill through 11 subcultures in preparations of chick embryo, monkey serum, and Tyrode's solution. The original inoculum was a 10% emulsion of infected mouse brain. The fourth and eighth subcultures in dilutions of 10^{-4} caused death in mice.

In view of the small number of culture generations and the absence of confirmation in vitro, the cultivation of this virus should be considered as only temporarily successful.

Embryonated Eggs.—Louping ill virus grew in embryonated eggs after chorioallantoic membrane, yolk sac, or intraembryonic inoculation (Edward,[326] Burnet,[327]

307. Footnote deleted.
308. Hirst, G. K.: Science **94**:22, 1941.
309. McClelland, L., and Hare, R.: Canad. J. Pub. Health **32**:530, 1941.
310. Cairns, H. J. F., and Edney, M.: J. Immunol. **69**:155, 1952.
311. von Magnus, P.: Acta path. et microbiol. scandinav. **28**:250, 1951; ibid. **28**:278, 1951.
312. Ziegler, J. E., and Horsfall, F. L.: J. Exper. Med. **79**:361, 1944.
313. Ziegler, J. E.; Lavin, G. I., and Horsfall, F. L.: J. Exper. Med. **79**:379, 1944.
314. Henle, W., and Henle, G.: Am. J. M. Sc. **207**:705, 1944. Footnote 76.
315. Isaacs, A., and Edney, M.: Australian J. Exper. Biol. & M. Sc. **28**:219, 1950; **28**:231, 1950.
316. Burnet, F. M., and Bull, D. R.: Australian J. Exper. Biol. & M. Sc. **21**:55, 1943.
317. Burnet, F. M., and Stone, J. D.: Australian J. Exper. Biol. & M. Sc. **23**:151, 1945.
318. Hirst, G. K.: J. Exper. Med. **86**:357, 1947.
319. Archetti, I., and Horsfall, F. L.: J. Exper. Med. **92**:441, 1950.
320. Archetti, I.: Proc. Soc. Exper. Biol. & Med. **80**:212, 1952.
321. Sugg, J. Y.: J. Bact. **58**:399, 1949.
322. Francis, T., and Salk, J. E.: Science **96**:499, 1942.
323. Stanley, W. M.: J. Exper. Med. **81**:193, 1945.
324. Friedewald, W. F.: J. Exper. Med. **80**:477, 1944.
325. Rivers, T. M., and Ward, S. M.: Proc. Soc. Exper. Biol. & Med. **30**:1300, 1933.
326. Edward, D. G. F.: Brit. J. Exper. Path. **28**:237, 1947.
327. Burnet, F. M.: Brit. J. Exper. Path. **17**:282, 1936.

Burnet and Lush [328]). The virus appeared in the blood stream following chorioallantoic membrane inoculation, and death of the embryo usually occurred in five to six days. Hepatic damage was marked. The embryo was shown to contain the highest concentration of virus when either the yolk sac or embryo stab mode of inoculation was employed.[326]

The ability to invade the central nervous system of sheep was apparently lost on extended egg passage, and subcutaneous injection of sheep with the modified egg culture virus induced immunity against subcutaneous but not intercerebral infection with fully virulent virus.[326]

LYMPHOCYTIC CHORIOMENINGITIS VIRUS

Tissue Cultures.—That the virus of lymphocytic choriomeningitis propagates readily in tissue culture was demonstrated by Casals-Ariet and Webster,[329] who isolated this agent as a contaminant from routine rabies cultures. The culture components consisted of mouse brain, monkey serum, and Tyrode's solution. Attention was first drawn to the possibility of a contaminating virus by the increased incubation period, the alteration of the disease syndrome, and the absence of rabies-immune serum protection when the tissue culture fluid, supposedly containing rabies virus, was inoculated into mice. The contaminant was carefully identified as the lymphocytic choriomeningitis virus after numerous mouse passages and Seitz filtrations. Of the two possible sources of contamination, the embryonic mouse tissue and the monkey serum, the latter was believed to be the actual source, in view of an outbreak of lymphocytic choriomeningitis in the monkey colony concurrent with the contamination.

MacCallum and Findlay,[330] in 1940, reported on a series of experiments begun in 1936. Two hundred seventy subcultures were carried out. These workers used a human serum, Tyrode's solution suspension of minced whole chick embryo for 97 passages; minced chick embryo brain was substituted for 18 cultures, and minced chick embryo minus brain and cord was utilized in the remainder of the subcultures. All cultures were incubated at 37 C., and transfers were performed every three to four days. The 25th and 50th subcultures had titers of 10^{-4}, and, although no attenuation of the pathogenicity of the virus for mice was observed during the tissue culture series, some changes in the character of the virus were apparent. A change in symptomatology in mice was noted when this host was inoculated with 66th subculture material, and a decrease in virulence for guinea pigs was recorded for virus from the 65th subculture. The original nature of the virus was regained after repeated guinea pig intracerebral passage.

Embryonated Eggs.—The virus of lymphocytic choriomeningitis was cultured through at least eight passages on the chorioallantoic membrane (Bengston and Wooley [331]). The virus was not lethal for the embryo, although the virus was present throughout the embryonated egg. No marked lesions could be demonstrated on the membrane.

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328. Burnet, F. M., and Lush, D.: Australian J. Exper. Biol. & M. Sc. **16**:232, 1938.

329. Casals-Ariet, J., and Webster, L. T.: J. Exper. Med. **71**:147, 1940.

330. MacCallum, F. O., and Findlay, G. M.: Brit. J. Exper. Med. **21**:110, 1940.

331. Bengston, I. A., and Wooley, J. G.: Pub. Health Rep. **51**:29, 1936.

LYMPHOGRANULOMA VENERUM VIRUS

Tissue Cultures.—Meyer and Anders [332] reported maintenance of the lymphogranuloma virus for six generations in flask preparations containing guinea pig kidney, testis, and guinea pig serum in Tyrode's solution. Material from the last culture injected into guinea pig inguinal areas caused inflammatory changes in about 50% of the inoculated animals. These cultures did not yield positive Frei tests when substituted for human pus.

Tamura [333] reported the presence of "peculiar granules" in supernatant fluid of guinea pig liver tissue cultures and positive Frei reactions with 23rd subculture fluids. Miyagawa and his co-workers [334] could not repeat the work of either Meyer and Anders or Tamura. Voet [335] also failed to substantiate Tamura's work, whereas D'Aunoy, von Haam, and Lichtenstein [336] reported partial confirmation. Miyagawa and co-workers [337] found that second culture generation of adult mouse testicle, brain, or spleen in hanging drop preparations infected with mouse brain virus produced skin reactions resembling those of the Frei test. This material was also infectious for mice. Malamos [338] reported inclusion bodies in rabbit cornea after 48 hours incubation. Also, the in vitro material caused typical symptoms in mice. Miyagawa inclusion bodies were observed by Manabe [339] in cultures of various organs of mouse embryo and of newborn mice. He obtained equally good growth in epithelial and fibroblast cells.

Gey and Bang,[340] in 1939, reported the maintenance of lymphogranuloma virus in continuous roller tube cultures of thyroid fibroblasts for seven months. Culture fluids killed mice and produced typical Frei test reactions in patients. Further, serum from Frei-positive patients was demonstrated to neutralize the infective agent in the tissue culture fluid. Characteristic granulocorpuscles were observed by these workers in hanging drop preparations when the plasma clot was dissolved before virus inoculation. After 111 days of continuous culture, virulence for mice was lost. Cytological observations and Frei test reactions suggested the presence of virus.

Sanders [83] cultured lymphogranuloma virus in ox serum ultrafiltrate cultures, using embryonic guinea pig brain as the tissue component. Cultures maintained at room temperature exhibited greater potency than those incubated at 37 C., and emphasis was placed on the method of obtaining material for the primary culture inoculum from early buboes. Two strains were carried for 12 and 21 generations, the original inoculum being diluted 18^{12} and 9.5^{21} times. No demonstrable virus was found in the supernatant fluid.

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332. Meyer, K., and Anders, H. E.: Klin. Wchnschr. **11**:318, 1932.

333. Tamura, J. T.: J. A. M. A. **103**:408, 1934.

334. Miyagawa, Y.; Mitamura, T.; Yaoi, H.; Ishii, N.; Okanishi, J.; Kanazawa, K., and Yamada, H.: Jap. J. Exper. Med. **14**:221, 1936.

335. Voet, J.: Compt. rend. Soc. biol. **118**:953, 1935.

336. D'Aunoy, R.; von Haam, E., and Lichtenstein, L.: Am. J. Path. **11**:737, 1935.

337. Miyagawa, Y.; Mitamura, T.; Yaoi, H.; Ishii, N.; Okanishi, J.; Goto, T., and Shimizu, S.: Jap J. Exper. Med. **14**:207, 1936.

338. Malamos, B.: Zentralbl. Bakt. (Abt. 1) **143**:1, 1938.

339. Manabe, K.: Jap. J. Exper. Med. **17**:333, 1939.

340. Gey, G. O., and Bang, F. B.: Bull. Johns Hopkins Hosp. **65**:393, 1939.

Two tissue culture techniques were employed successfully by Takemori,[341] who observed lymphogranuloma virus propagation in agar cultures of chick embryo and mouse testicle and also in deep suspended cell cultures of mouse and chick embryo tissues. Twenty-three passages were carried out with one strain of virus in the agar slant tissue culture. Virus grew readily in the deep (2.4 cm.) cultures, attaining titers of 10^{-4} to 10^{-5} after 7 to 10 days' incubation at 37 C. Granulocorpuscles were observed, and the virus multiplied under anaerobic condition but failed to do so in the absence of glucose.

Embryonated Eggs.—The lymphogranuloma virus propagated in the embryonated egg following chorioallantoic membrane or yolk sac inoculation (Miyagawa and co-workers [342]; Rake, McKee, and Shaffer [343]). The latter route was considerably more effective.[343] Two strains of lymphogranuloma were isolated directly from inguinal buboes into the yolk sac (Shaffer and co-workers [344]). Yolk sac inoculation induced the production of satisfactory Frei-test antigen (Grace, Rake, and Shaffer [345]; Howard and Hull [346]), whereas the results with chorioallantoic membrane inoculation were irregular. The morphologic development and growth rate of virus particles in the yolk sac were investigated (Rake and Jones [347]). A toxin was found in low concentrations in infected yolk sac (Rake and Jones [348]). The appearance of lesions on the chorioallantoic membrane was not regular, and large doses of virus were required to kill the embryo by this route (Howard and Hull,[346] Melczer and Sipos [349]). Yolk sac inoculation, however, caused death in almost all infected eggs before the 15th day.[347]

MEASLES VIRUS

Tissue Cultures.—Rake, Shaffer, and Jones [350] reviewed the attempts to propagate the virus of measles and concluded that, of previous investigators, Plotz [351] alone appeared to have cultivated the virus. Plotz cultured the virus in proliferating cell preparations for 10 passages with a 7^{-10} dilution of the original inoculum. Five milliliters of 10th culture material injected subcutaneously produced measles in a monkey. Enders and Hammon [352] used Plotz's methods but doubted whether the symptoms observed in one monkey were indicative of a specific infection.

Rake and his co-workers also followed Plotz's technique. Cultures of minced chick embryo in a fluid medium of Tyrode's solution, monkey serum, and chicken

341. Takemori, N.: Kitasato Arch. Exper. Med. **21**:261, 1948.

342. Miyagawa, Y.; Mitamura, T.; Yaoi, H.; Ishii, N., and Okanishi, J.: Jap. J. Exper. Med. **13**:733, 1935.

343. Rake, G.; McKee, C. M., and Shaffer, M. F.: Proc. Soc. Exper. Biol. & Med. **43**:332, 1940.

344. Shaffer, M. F.; Jones, H.; Grace, A. W.; Hamre, D. M., and Rake, G.: J. Infect. Dis. **75**:109, 1944.

345. Grace, A. W.; Rake, G., and Shaffer, M. F.: Proc. Soc. Exper. Biol. & Med. **45**:259, 1940.

346. Howard, M. E., and Hull, W. S.: J. Infect. Dis. **68**:73, 1941.

347. Rake, G., and Jones, H. P.: J. Exper. Med. **75**:323, 1942.

348. Rake, G., and Jones, H. P.: J. Exper. Med. **79**:463, 1944.

349. Melczer, M., and Sipos, K.: Acta dermat.-venereol. **22**:320, 1941.

350. Rake, G.; Shaffer, M. F., and Jones, H. P.: J. Infect. Dis. **69**:65, 1941.

351. Plotz, H.: Bull. Acad. de méd., Paris **119**:598, 1938.

352. Enders, J. F., and Hammon, W. M.: Symposium on Virus and Rickettsial Diseases, Cambridge, Mass., Harvard University Press, 1940, p. 237.

plasma were infected with ether-treated throat washings from children in the early stages of the disease. Tenth passage material was injected subcutaneously and intranasally into a Macacus mulattus monkey and produced typical measles and a subsequent solid immunity. The retention of infectivity in a tissue culture fluid after a dilution of 1 : 282,500,000 of the original inoculum strongly suggests the propagation of the measles virus in this system.

Embryonated Eggs.—Measles virus was cultivated on the chorioallantoic membrane of 8 to 16-day-old embryonated eggs (Rake and Shaffer; Hurst and Cooke [353]). Sixty-six passages were carried out; material from most of these produced mild measles symptoms in monkeys (Stokes and co-workers [354]). No specific, regularly occurring microscopic lesions were demonstrated, but necrosis was evident in the embryo liver.[355] Although preliminary tests of an egg culture vaccine were encouraging, later field trials were disappointing (Stokes and co-workers [354]; Stokes [356]).

MENINGOPNEUMONITIS VIRUS

Embryonated Eggs.—The virus of meningopneumonitis was successfully maintained on the chorioallantoic membrane (Francis and Magill [357]), in the yolk sac (Rake and Jones [358]), and in the allantoic cavity (Sigel, Girardi, and Allen [359]). A toxin was demonstrated in the yolk sac.[358] Development of the virus in the embryonated egg was fully described and, in general, resembled the growth of other psittacosis-lymphogranuloma group viruses (Weiss [360]).

MOUSE ENCEPHALOMYELITIS AND MOUSE ENCEPHALOMYOCARDITIS VIRUSES

Tissue Cultures.—The viruses of mouse encephalomyelitis and mouse encephalomyocarditis groups appear to propagate readily in suspended cell cultures containing salt solution-serum ultrafiltrate.

Sanders and Jungeblut [7] carried out 200 successive passages of minced embryonic mouse brain cultures infected with Col-SK viruses. These investigators found that embryonic brain gave titers 100 times higher than whole mouse embryo minus brain or minus brain and cord. When the virus was propagated through 49 tissue culture generations, a loss of peripheral virulence occurred.[7] Shean and Schultz [361] observed propagation of the Col-SK virus in the presence of embryonic mouse brain, lung, and small intestine; in the presence of whole chick embryo, and, to a less degree, in the presence of mouse embryo tail.

The MM virus was found to multiply in embryonic mouse brain, heart, and tail cultures, and lower titers were obtained in mouse embryo lung and whole chick

353. Rake, G., and Shaffer, M. F.: Nature, London **144**:672, 1939; J. Immunol. **38**:177, 1940. Hurst, E. W., and Cooke, B.: M. J. Australia **1**:323, 1941.

354. Stokes, J.; O'Neill, G. C.; Shaffer, M. F.; Rake, G., and Maris, E. P.: J. Pediat. **22**:1, 1943.

355. Footnote 353. Footnote 354.

356. Stokes, J.: Personal communication to Beveridge and Burnet.[5]

357. Francis, T., and Magill, T. P.: J. Exper. Med. **68**:147, 1938.

358. Rake, G., and Jones, H.: J. Exper. Med. **79**:463, 1944.

359. Sigel, M. M.; Girardi, A. J., and Allen, E. G.: J. Exper. Med. **94**:401, 1951.

360. Weiss, E.: J. Infect. Dis. **84**:125, 1949.

361. Shean, D. B., and Schultz, E. W.: Proc. Soc. Exper. Biol. & Med. **73**:622, 1950.

embryo.[361] Chambers, Smith, and Evans [362] used minced whole mouse embryo, minced embryonic mouse intestine, and minced hamster bladder for the propagation of the MM virus. In plasma clot preparations embryonic mouse intestine did not support virus multiplication, and subcutaneous tissue and skeletal muscle of the hamster were also unsatisfactory. Both 7-month mouse testicle and 57 to 58-year human testicle cultures gave evidence of virus multiplication, but in each case the titers were low.[363]

Parker and Hollender [364] reported 20 passages of Theiler's GD VII mouse encephalomyelitis virus, and Pearson [365] achieved 45 passages with the same virus. The former workers employed embryonic mouse brain. Pearson used one-day-old mouse brain and was also able to make 26 serial passages of the GD VII virus in minced seven- to eight-day chick embryo brain. Shean and Schultz [361] carried the virus for 15 passages in mouse embryo brain, mouse embryo tail, and whole chick embryo. The FA strain of Theiler's virus propagated only in embryonic mouse brain and whole chick embryo cultures.[361]

With relatively large inocula, Col-SK virus attained maximum titers in 19 hours when incubated at 37 C.[7] At pH 7 to 7.4, maximum titers of GD VII virus strain were observed in two to three days.[365] Both viruses were reported to multiply at a slower rate when the initial inoculum was near to, or less than, the minimum lethal mouse dose.[366] The MM virus exhibited a one-hundred-fold increase in potency after two days of incubation and in one experiment a ten-thousand-fold increase in four days when the virus inoculum did not exceed 1 MLD.

The effect of pH was investigated by Sanders and Jungeblut [7] and by Pearson.[365] The Col-SK virus appears from a comparison of the data to be more sensitive to pH change than the somewhat resistant GD VII virus. Parker and Hollender [364] observed cultures incubated at 35 C. to give titers of GD VII virus 1,000 times higher than those incubated at 37.5 C. Pearson found temperatures ranging from 31 to 37 C. to give equivalent titers, but at 25 C. and 38 C. the titers did not exceed 10^{-2}. The Col-SK virus was of equal titer when incubated at 37 C. or room temperature, but, under the latter condition, a four-day lag period was encountered before virus propagation began.[7]

Embryonated Eggs.—Col-SK (Enright and Schultz [367]; Schultz and Enright [368]; Verlinde, Waller-Fetter, and deBaan [369]), C(M),[370] MM,[370] Mengo (Dick,[371] Taylor [184]), and Theiler's (Gard,[372] Riordan and Sa Fleitas,[373] Dunham and

362. Chambers, V. C.; Smith, W. M., and Evans, C. A.: J. Immunol. **65**:605, 1950.
363. Chambers, V. C.; Smith, W. M., and Evans, C. A.: Proc. Soc. Exper. Biol. & Med. **76**:213, 1951.
364. Parker, R. C., and Hollender, A. J.: Proc. Soc. Exper. Biol. & Med. **60**:88, 1945.
365. Pearson, H. E.: J. Immunol. **64**:447, 1950.
366. Footnote 7. Footnote 365.
367. Enright, J. R., and Schultz, E. W.: Proc. Soc. Exper. Biol. & Med. **66**:541, 1947.
368. Schultz, F. W., and Enright, J. B.: Proc. Soc. Exper. Biol. & Med. **63**:8, 1946.
369. Verlinde, J. D.; Waller-Fetter, P., and De Baan, P.: Leeuwenhoekned. Tijdschr. **17**: 183, 1951.
370. Footnote 367. Footnote 369.
371. Dick, G. W. A.: Proc. Soc. Exper. Biol. & Med. **73**:77, 1950.
372. Gard, S.: Acta med. scandinav., Supp. 143, p. 1, 1943; Nature, London **152**:660, 1943.
373. Riordan, J. T., and Sa Fleitas, M. J.: Science **103**:499, 1946; J. Immunol. **56**:263, 1947.

Parker [374]) viruses were grown in embryonated eggs. The Col-SK virus was inoculated successfully on the chorioallantoic membrane, in the yolk sac, and in the embryo.[368] The Mengo virus propagated in serial passage by yolk sac inoculation.[371] The MM virus presented more difficulties in culture. Several attempts to pass the virus on the chorioallantois were unsuccessful beyond the sixth transfer (Brutsaert, Jungeblut, and Knox [375]; Jungeblut and Dalldorf [376]; Jungeblut [377]); in another laboratory the chorioallantoic membrane was shown to support virus growth.[367] Amniotic fluid continued to contain virus and, in fact, increased in mouse infectivity.[369] The C(M) virus grew on the chorioallantoic membrane.[367]

The GD VII strain of Theiler's virus was passed through eggs on the chorioallantois [378]; the FA strain propagated when inoculated via the chorioallantoic membrane, yolk sac, or allantoic cavity.[373]

The mouse encephalomyelitis and mouse encephalomyocarditis viruses were lethal for embryos but produced no distinctive microscopic lesions. Low-titer hemagglutinins were demonstrated in Col-SK-infected embryo brain.[369]

MOUSE PNEUMONITIS VIRUS

Embryonated Eggs.—Mouse pneumonitis virus did not propagate satisfactorily on the chorioallantoic membrane or in the allantoic cavity (Nigg and Eaton [379]). Passage through the yolk sac, in contrast, was readily initiated and Giemsa-staining bodies were found in large numbers.[379] The virus was lethal for 6-day chick embryos in six to seven days. A virus indistinguishable from psittacosis but occurring naturally in mice grew in chorioallantoic membrane, producing psittacosis-like pocks (DeBurgh, Jackson, and Williams [380]). This virus was also demonstrated to multiply in the amniotic cavity, in the yolk sac, and in the allantoic cavity after a preliminary yolk sac passage.

MUMPS VIRUS

Tissue Cultures.—Weller and Enders,[6] who first reported propagation of the mumps virus in tissue cultures, employed the suspended cell technique. These workers used minced amnion in a fluid medium of serum ultrafiltrate and Hanks' solution (1:3). The supernatant fluid was replaced at four-day intervals, and, while the virus was subcultured for only two generations, both hemagglutination and egg infectivity tests gave evidence of extensive multiplication of the egg-adapted strain of virus in the cultures. In Chart 1 are presented the classical growth curves obtained in these experiments; the increase in hemagglutinin was calculated at $3.3 \times 10.^{14}$

Cushing and Morgan [381] and Kilham and Murphy [382] adapted Earle's methods of tissue culture on cellophane to the propagation of the mumps virus. The former

374. Dunham, W. B., and Parker, S.: J. Bact. **45**:80, 1943.
375. Brutsaert, P.; Jungeblut, C. W., and Knox, A.: J. Pediat. **29**:350, 1946.
376. Jungeblut, C. W., and Dalldorf, G.: Am. J. Pub. Health **33**:169, 1943.
377. Jungeblut, C. W.: J. Exper. Med. **81**:275, 1945.
378. Footnote 374. Footnote 372.
379. Nigg, C., and Eaton, M. D.: J. Exper. Med. **79**:497, 1944.
380. DeBurgh, P.; Jackson, A. V., and Williams, S. E.: Australian J. Exper. Biol. & M. Sc. **23**:107, 1945.
381. Cushing, R. T., and Morgan, H. R.: Proc. Soc. Exper. Biol. & Med. **79**:497, 1952.
382. Kilham, L., and Murphy, H. W.: Proc. Soc. Exper. Biol. & Med. **80**:495, 1952.

workers used minced amnion covered with a perforated cellophane disc in the study of the effect of certain vitamin analogues on virus growth. Results similar to those of Weller and Enders were obtained with respect to the time of the maximum hemagglutinin titers and also the persistence of the virus in tissue culture. Kilham and Murphy grew the mumps virus on comminuted mouse embryo evenly distributed under a sheet of perforated cellophane in a T-60 flask. A nutrient fluid consisting of horse serum, balanced saline, and embryo extract supported virus propagation, whereas serum ultrafiltrate did not. With these cultures, although egg infectivity was observed in dilutions as high as 10^{-4}, no hemagglutinin could be demonstrated. A greatly increased ability to grow in suckling mouse brain was noted after 20 weekly culture passages when the transfer material contained cell debris, as well as fluid. The tissue culture virus remained nonpathogenic for mice, and attempts to propagate the mumps virus in various suckling and adult mouse tissues, including placenta, uterus, spleen, and liver, were unsuccessful.

Watson,[383] employing fluorescein-labeled antibodies, demonstrated the virus in suspended cell cultures to be restricted to the cytoplasm of the cells which came in contact with the inoculum.

Embryonated Eggs.—The first definite propagation of mumps virus in the embryonated egg was achieved in 1945 by Habel,[384] who inoculated virus from parotid glands of mumps-infected monkeys in the yolk and amniotic sacs and allantoic cavity. After 10, 11, and 12 passages, the presence of virus was demonstrated by means of complement-fixing antigen. Early passage virus was shown to produce parotitic swelling and fever in monkeys.

The amniotic route of inoculation was found better than the yolk sac route, while the allantoic route was found to yield the poorest result [384] (Beveridge, Lind, and Anderson [385]). Inoculation on the chorioallantoic membrane produced no specific lesions, and death of the embryo was irregular, but antigen was demonstrable. Yolk sac inoculation caused the death of 50% of the embryos in 7 to 12 days, with petechial hemorrhages in the skin and yolk sac. No pathology, gross or microscopic, could be observed after amniotic inoculation, and the embryos survived.

A fowl red blood cell agglutinin was demonstrated in amniotic fluid (Levens and Enders [386]), but allantoic fluid was found to inhibit red blood cell agglutination (Beveridge and Lind [387]). A factor that is hemolytic for human, chicken, and sheep cells was shown to be present in the amniotic and allantoic fluid of mumps-infected eggs (Morgan, Enders, and Wagley [388]; Chu and Morgan [389]). In one laboratory (Enders and co-workers [390]), 25 serial passages caused the virus to lose its ability to infect monkeys, although immunizing effects, as well as complement-fixing antigens and antihemagglutinin factors, remained; another laboratory reported no change in virus characteristics after 30 passages in the eggs (Habel, personal communication, Enders and co-workers [390]). An effective vaccine from embryonated

383. Watson, B. K.: Proc. Soc. Exper. Biol. & Med. **79**:222, 1952.
384. Habel, K.: Pub. Health Rep. **60**:201, 1945.
385. Beveridge, W. I. B.; Lind, P. E., and Anderson, S. G.: Australian J. Exper. Biol. & M. Sc. **24**:15, 1946.
386. Levens, J. H., and Enders, J. F.: Science **102**:117, 1945.
387. Beveridge, W. I. B., and Lind, P. E.: Australian J. Exper. Biol. & M. Sc. **24**:127, 1946.
388. Morgan, H. R.; Enders, J. F., and Wagley, P. F.: J. Exper. Med. **88**:503, 1948.
389. Chu, L. W., and Morgan, H. R.: J. Exper. Med. **91**:393, 1950.
390. Enders, J. F.; Levens, J. H.; Stokes, J., and Berenberg, W.: J. Immunol. **54**:283, 1946.

eggs infected with mumps virus has been prepared (Beveridge and Lind [387]; Muntz, Powell, and Culbertson [391]; Habel [392]).

NEWCASTLE DISEASE VIRUS

Tissue Cultures.—Topacio [393] cultivated a strain of Newcastle disease virus for 31 culture generations over a period of 112 days in Carrel flasks containing chick embryo tissue, Tyrode's solution, and chick plasma. Each subculture was diluted 1:30, and the final subculture was infective for chickens. Subinfective doses of culture virus were satisfactory as a vaccine. Bankowski [394] employed the suspended cell technique to carry the virus through 50 culture generations. In the first studies (Bankowski and Boynton [395]) liver and heart of 10 to 13-day chick embryos were used. However, it was later demonstrated [394] that the virus propagated as readily in a medium containing minced whole embryos. The tissues were suspended in either bovine or chicken serum ultrafiltrate. A loss of the capacity for chicken blood cell agglutination was noted in early passages, but embryo pathogenicity was retained, and allantoic fluid of killed embryos contained hemagglutinin.[395] Virus cultivated in the presence of bovine serum ultrafiltrate steadily decreased in virulence for chickens; 40th to 50th passage subcultures failed to produce symptoms in fowl.[394] The culture virus was still capable of immunizing chickens against 200,000 MLD.[395] Laboratory and field vaccination trials with this attenuated strain of virus administered by the air-borne route to chickens were highly encouraging.[394] The modification of the virus in the chicken serum ultrafiltrate cultures was somewhat less marked. [396] The virus did not propagate on Ehrlich tumor tissue in vitro (Moore and co-workers[397]).

Although the successful cultivation of the Newcastle disease virus has been reported by only a few workers, it has been placed in Group I for the following reasons. The results have been consistent and essentially agree with the embryonated egg experience. There appears to be no difficulty in initiating and maintaining the cultures; the reports have been comprehensive, and the virus has been observed for significant periods. The economic hazards associated with bringing this agent into laboratories make it appear unlikely that many investigators will pursue the subject.

Embryonated Eggs.—Newcastle disease virus multiplied in the embryonated egg causing death when inoculated on the chorioallantoic membrane, intra-allantoically, intra-amniotically, and intravenously (Burnet and Ferry,[398] Burnet [399]). The course of infection in the egg was quite similar to that of influenza. Chorioallantoic membrane lesions were indistinguishable from the changes in the membrane caused by adapted influenza virus, and amniotic and allantoic fluid of infected egg were found

391. Muntz, H. H.: Powell, H. M., and Culbertson, C. G.: J. Lab. & Clin. Med. **34**:199, 1949.
392. Habel, K.: Am. J. Hyg. **54**:295, 1951.
393. Topacio, T.: Philippine J. Sc. **53**:245, 1934.
394. Bankowski, R. A.: Vet. Med. **45**:322, 1950.
395. Bankowski, R. A., and Boynton, W. H.: Vet. Med. **43**:305, 1948.
396. Footnote 394. Footnote 395.
397. Moore, A. G.; Diamond, L. C.; Mackay, H. H., and Sabachewsky, L.: Proc. Soc. Exper. Biol. & Med. **81**:498, 1952.
398. Burnet, F. M., and Ferry, J. D.: Brit. J. Exper. Path. **15**:56, 1934.
399. Burnet, F. M.: Australian J. Exper. Biol. & M. Sc. **20**:81, 1942.

to contain a fowl red blood cell agglutinin. In one instance (Iyer and Dobson [400]) after 33 generations and in another [398] after 12 generations in the embryonated egg, the virulence of the virus for birds was lost, but its antigenicity was retained.

There have been several detailed microscopic examinations of the egg-adapted virus (Bang [401]; Reagan, Hickman, Lillie, and Brueckner [402]). Purified chorioallantoic fluid virus (Cunha and co-workers [403]) and the growth rate (Gordon, Birkeland, and Dodd [404]) have been studied. A vaccine was prepared from duck embryo passage material (Clancy, Cox, and Bottorff [405]; Komarov and Goldsmit [406]; Markham, Cox, and Bottorff [407]).

PACHECO'S PARROT DISEASE VIRUS

Embryonated Eggs.—The virus of Pacheco's parrot disease was passed for six generations on the chorioallantois (Rivers and Schwentker [408]). Distinct opaque foci on the membrane and occasional white spots in the liver and spleen were observed microscopically. Embryo deaths occurred in three to five days after inoculation, and histologic examination revealed ectodermal proliferation and necrosis, in addition to necrosis of visceral organs. Inclusion bodies in these organs were also noted. The final, i. e., the sixth, egg generation was infectious for parrots.

POLIOMYELITIS VIRUS

Tissue Cultures.—It is in the field of poliomyelitis cultivation that there has been the greatest activity, and it is here that greatest success has been achieved in recent years. Whereas poliomyelitis growth in vitro was considered questionable in 1939, it can now be considered one of the viruses grown at will.

Prior to the work of Enders and his associates (Enders and co-workers,[409] Weller and co-workers,[410] Robbins and co-workers [91]), in vitro results were irregular and controversial. Included in the early experiments were attempts to propagate poliomyelitis in infected monkey spinal ganglia (Levaditi [411]), rabbit kidney plus human ascitic fluid (Flexner and Noguchi,[412] Long and co-workers [413]), and embryonic chick brain in monkey serum-Tyrode's solution (Gildemeister [414]).

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400. Iyer, S. G., and Dobson, N.: Vet. Rec. **52**:889, 1940.
401. Bang, F. B.: Proc. Soc. Exper. Biol. & Med. **64**:135, 1947; J. Exper. Med. **88**:251, 1948.
402. Reagan, R. L.; Hickman, J. W.; Lillie, M. G., and Brueckner, A. L.: J. Infect. Dis. **85**:256, 1949.
403. Cunha, R.; Weil, M. L.; Beard, D.; Taylor, A. R.; Shaup, D. G., and Beard, J. W.: J. Immunol. **55**:69, 1947.
404. Gordon, L. E.; Birkeland, J. M., and Dodd, M. C.: Proc. Soc. Exper. Biol. & Med. **80**:205, 1952.
405. Clancy, C. F.; Cox, H. R., and Bottorff, C. A.: Poultry Sc. **28**:58, 1949.
406. Komarov, A., and Goldsmit, L.: Cornell Vet. **37**:368, 1947.
407. Markham, F. S.; Cox, H. R., and Bottorff, C. A.: Poultry Sc. **28**:52, 1949.
408. Rivers, T. M., and Schwentker, F. F.: J. Exper. Med. **55**:911, 1932.
409. Enders, J. F.; Weller, T. H., and Robbins, F. C.: Science **109**:85, 1949.
410. Weller, T. H.; Robbins, F. C., and Enders, J. F.: Proc. Soc. Exper. Biol. & Med. **72**:153, 1949.
411. Levaditi, C.: Compt. rend. Soc. biol. **75**:202, 1913.
412. Flexner, S., and Noguchi, H.: J. Exper. Med. **18**:461, 1913.
413. Long, P. H.; Olitsky, P. K., and Rhodes, C. P.: J. Exper. Med. **52**:361, 1930.
414. Gildemeister, E.: Zentralbl. Bakt. (Abt. 1) **109**:284, 1933.

Sabin and Olitsky [415] probably were the first to cultivate the poliomyelitis virus in 1936 in the nervous tissue taken from a human embryo and minced in Tyrode's solution. One hundred milligrams of tissue in 4.5 ml. of Tyrode's solution was incubated at 37 C. in 50 cc. Erlenmeyer flasks and inoculated with 0.5 ml. of filtrate from a 5% poliomyelitis cord suspension. Titration of the original inoculum revealed it to contain 50 MLD. Three and six subcultures were made, and inoculation of final culture material produced a characteristic disease in monkeys.

In 1949, Enders and co-workers [409] not only confirmed the findings of Sabin and Olitsky with the Lansing strain but were also able to propagate the virus in human embryonic non-neural tissues. Since that time numerous confirmatory reports have appeared in rapid succession, and the three immunologic types of poliomyelitis have been propagated in human and monkey neural and non-neural tissues.

Tissues: Cells of widely divergent types, when maintained successfully in tissue culture, have been found to satisfy the growth requirements of poliomyelitis virus. A summary [416] of the tissues which have been investigated is presented in Table 1. An attempt has been made to coordinate present opinion in regard to the ability of different cells to support poliomyelitis propagation. Special attention should be paid to the contributions of Enders, Weller, and Robbins,[417] who first noted the capacity of the virus to grow in the presence of non-neural tissue, and to the efforts of Smith, Chambers, and Evans,[416f] who grew the virus in human and monkey testicular tissue.[418] Although monkey testicle has become a popular substrate, it is still not clear which cellular elements in the testes are necessary for virus propagation.[418] Morphologically pure cultures of fibroblasts from Macaca cynomolgus monkey testicle support growth of Y-SK virus. According to Scherer and Syverton [419] and Ledinko and co-workers,[416k] other cells of the monkey testicular tissue play a part in the process of virus multiplication.

It is interesting that, although human and monkey testicular tissues readily support the multiplication of poliomyelitis virus, testicular tissues of mice, guinea pigs, hamsters, rabbits, or bulls do not (Smith and co-workers,[27] Robbins and co-workers [28]). Testicular tissue from both immune and nonimmune M. rhesus and M. cynomolgus monkeys are equally susceptible (Ledinko and co-workers [416o]).

Chick, mouse, and beef embryonic tissues do not support the propagation of poliomyelitis virus; likewise, the tissues from these mature animals do not support its propagation (Weller and co-workers,[416b] Robbins and co-workers,[28] Duncan and co-workers [60]). Other tissues found not to support the multiplication of this virus are human embryonic liver and rabbit kidney (Weller and co-workers [416b]).

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415. Sabin, A. B., and Olitsky, P. K.: Proc. Soc. Exper. Biol. & Med. **34**:357, 1936.

416. (*a*) Milzer, A.; Levinson, S. O.; Vanderboom, K., and Adelman, P.: Proc. Soc. Exper. Biol. & Med. **74**:136, 1950. (*b*) Weller, T. H.; Enders, J. F.; Robbins, F. C., and Stoddard, M. B.: J. Immunol. **69**:645, 1952. (*c*) Footnote 410. (*d*) Footnote 93. (*e*) Footnote 28. (*f*) Smith, W. M.; Chambers, V. C., and Evans, C. A.: Northwest Med. **49**:368, 1950. (*g*) Footnote 27. (*h*) Syverton, J. T.; Scherer, W. F., and Butorac, G.: Proc. Soc. Exper. Biol. & Med. **77**:23, 1951. (*i*) Footnote 41. (*j*) Salk, J.: J. A. M. A. **151**:1081, 1953. (*k*) Ledinko, N.; Riordan, J. T., and Melnick, J. L.: Am. J. Hyg. **55**:323, 1952. (*l*) Melnick, J. L., and Riordan, J. T.: Proc. Soc. Exper. Biol. & Med. **81**:208, 1952. (*m*) Youngner, J. S.; Ward, E. N., and Salk, J. E.: Am. J. Hyg. **55**:291, 1952; (*n*) ibid. **55**:301, 1952. (*o*) Ledinko, N., and Melnick, J. L.: Proc. Soc. Exper. Biol. & Med. **81**:144, 1952.

417. Footnote 91. Footnote 409. Footnote 410.

418. Footnote 27. Footnote 416*f*.

419. Scherer, W. F., and Syverton, J. T.: J. Exper. Med. **96**:369, 1952; ibid. **96**:389, 1952.

In the preparation of tissues for poliomyelitis cultures, the usual tissue culture procedures are followed insofar as careful mincing and washing of tissue fragments are concerned. However, it is important to note that occasionally tissue from a

TABLE 1.—*Tissues Which Support Poliomyelitis Propagation*

Tissue	Virus Type	References *
Human Embryonic		
	RMV	Sabin [415]
	Lansing	Enders,[409] Franklin,[57] Milzer,† [416a] Thicke,[58] Weller † [416b, c]
	Brunhilde	Weller [416b, c]
Brain and spinal cord	Leon	Weller [416b]
	Brunhilde	Robbins,[416d, e] Svedmyr,[12] Weller [416b, c]
Skin-muscle	Lansing	Enders,[409] Milzer,[416a] Robbins,[29] Svedmyr,[12] Thicke,† [58] Weller [416b]
	Leon	Robbins,[416d, e] Weller [416b]
	Brunhilde	Svedmyr,[12] Weller [416b]
Intestine	Lansing	Enders,[409] Milzer,† [416a] Svedmyr,[12] Thicke,† [58] Weller [416b]
Adrenal	Lansing	Weller [416b]
Kidney	Lansing	Thicke,[58] Weller,[416b] Franklin [57b]
Thyroid	Lansing	Weller [416b]
Lung	Lansing	Thicke,[58] Weller † [416b]
Spleen	Lansing	Weller † [416b]
Heart	Lansing	Weller [416b]
Thymus	Lansing	Thicke † [58]
Human Nonembryonic		
Foreskin (boys, 3 mo. to 11 yr.)	Brunhilde	Weller ‡ [416b]
	Lansing	Milzer,‡ [416a] Robbins,[29] Smith,† [416f] Weller † [416b]
Testicle	Brunhilde	Smith [416f, g]
	Lansing	Robbins,[29] Smith,[416f, g] Syverton,[416b, i] Weller [416b]
	Brunhilde	Robbins,[416d, e] Svedmyr,[12] Weller,[416b] Salk [416j]
Kidney (infants and children)	Lansing	Robbins,[29] Svedmyr,[12] Weller,[416b] Salk [416j]
	Leon	Robbins,[416d, e] Weller,[416b] Salk [416j]
	Brunhilde	Robbins [29] (roller tubes), Svedmyr [12]
Uterus	Lansing	Robbins,[29] Svedmyr,[12] Weller ‡ [416b] (failure with suspended cell; success with roller tube cultures)
	Leon	Robbins [29] (roller tubes)
Placenta	Lansing	Milzer,† [416a] Thicke,† [58] Weller,† [416b] Smith ‡ [416f] (using amnion)
Tonsil	Lansing	Smith,† [416f] Franklin [57b]
Thyroid	Lansing	Weller [416b]
Tumors (embryoma of kidney, neuroblastoma)	Lansing	Weller [416b]
Monkey		
	Brunhilde	Ledinko,[416k] Melnick,[416l] Syverton,[41] Wood,[59] Youngner [416m, n]
Testicle	Lansing	Duncan,[60] Ledinko,[416k, o] Robbins,[29] Smith,[97] Syverton,[416b, i] Thicke,[58] Wood,[59] Youngner [416m]
	Leon	Wood,[59] Youngner [416m]
Kidney	Lansing	Duncan,[60] Ledinko,[416k] Robbins,[24] Weller [416b]
	Leon	Robbins [24]
Muscle	Lansing	Duncan,[60] Ledinko [416k]
Brain and cord	Lansing	Duncan [60]
Lung	Lansing	Duncan [60]
Intestine	Lansing	Duncan [60]

* Only the first author's name has been used in all references.
† Multiplication of poliomyelitis virus was questionable.
‡ No multiplication of poliomyelitis virus.

monkey may unexpectedly fail to support propagation of the virus. This observation made by Ledinko and Melnick [416o] is interpreted as perhaps being due to an unusual genetic factor, a latent virus, or some other undetermined cause. Whatever

the reason, in preparing tissues for poliomyelitis cultures it is apparently best to use a pool from several animals.

The Boston workers (Enders,[420] Weller and co-workers,[416b] Robbins and co-workers [28]) have recently published the first of a series of communications comprehensively discussing the problem of poliomyelitis propagation in vitro. In these papers the technical details of both the suspended cell and the roller tube cultures are presented. They have noted that storage of minced human embryonic skin-muscle is feasible for 28 days at 5 C. when the tissues are maintained in a mixture of Hanks' salt solution-serum ultrafiltrate containing 0.2% human albumin. Other workers were able to store monkey testicular tissue for six or seven days at 4 C. (Youngner and co-workers,[416m] Syverton and Scherer [41]).

In relation to the tissue fluid ratio, the general rules mentioned in the Introduction seem to apply to poliomyelitis propagation, i. e., the range is approximately between 1:100 [421] to 1:500.[422] On the basis of additional experiments, Ledinko and her associates [416k] have expressed the opinion that the amount of tissue has no effect on the amount of virus liberated into the fluid phase "within the limits tested."

Media: To isolate and identify strains of poliomyelitis virus, it is desirable to obtain an initial luxuriant outgrowth of fibroblasts. Any medium which will stimulate active cellular proliferation may be used. A balanced salt solution, chick embryo extracts, and horse serum in various amounts have been employed in many laboratories. According to Robbins, Weller, and Enders,[28] beef embryo extract produces better cellular proliferation in human embryonic skin-muscle preparations. In a recent report Enders [61] has recommended bovine amniotic fluid.

Melnick and Riordan [416l] have reported that a medium consisting of lactalbumin hydrolysate, balanced salt solution, and serum ultrafiltrate fosters more rapid outgrowth of fibroblasts from monkey testicular tissue than does a medium containing chick embryo extract. In addition, virus growth was supported as well and apparently for longer periods in the hydrolysate tubes than in the chick embryo extract tubes.

For maintenance of serial poliomyelitis cultures, ox serum ultrafiltrate usually replaces the whole serum.

More recently, the use of synthetic mixture No. 199 has come into prominence as a nutrient vehicle for poliomyelitis propagation (Franklin and co-workers,[57] Wood and co-workers,[59] Thicke and co-workers,[58] Duncan and co-workers [60]). This is particularly interesting since the originators of the synthetic medium (Morgan, Morton, and Parker [16]) considered it inadequate for continuous tissue culture studies. However, in the hands of the Canadian investigators, this medium appears to show promise for all phases of poliomyelitis in vitro research. They emphasize the important fact that this medium supports cellular metabolism and virus multiplication for periods of more than 100 days.[423]

Conditions of Incubation: Through the study of the growth curve of poliomyelitis virus it has been determined that maximum titers are not reached as

420. Enders, J. F.: J. Immunol. **69**:639, 1952.
421. Footnote 409. Footnote 416*f*.
422. Footnote 41. Footnote 416 *h*.
423. Footnote 60. Footnote 57 *b*.

rapidly as with many other viruses (Ledinko and co-workers,[416k] Scherer and Syverton [419]). Thus, it is important to allow sufficient time before subcultures are prepared (Enders [420]). The fluid is usually changed every 3 to 5 days and subcultures prepared every 8 to 20 days. However, when the well-buffered synthetic medium No. 199 is used, such frequent fluid changes are not necessary (Thicke and co-workers,[58] Wood and co-workers [59]). Further, in regard to conditions of incubation, it should be noted that the published reports have given 35 to 37 C. as the temperature of incubation.

Cytopathogenic Phenomenon: Early in the studies of the cultivation of Lansing poliomyelitis virus in suspended cell cultures of human nervous and non-nervous tissue, Enders and co-workers [409] noted significant degenerative changes in the virus-infected cells. Subsequently (Robbins and co-workers [91]) these observations were extended to include the Brunhilde strain, and the term "cytopathogenic phenomenon" was applied to the visible culture changes, which included cellular degeneration, failure of explants to proliferate, and a reflection of the process in abnormal pH differences between infected and uninfected cultures. A note of caution has been introduced in the evaluation of the cytopathogenic phenomenon to the effect that cellular changes are more dependable than pH changes (Robbins and co-workers,[28] Youngner and co-workers [416n]).

In the first studies, the cytopathogenic phenomenon was demonstrated only after approximately 30 days of virus cultivation. Later, an improved method was developed employing the hanging drop cultures.[91] The best results for cytopathogenic work are found with the roller tube technique currently used in most laboratories.

The cytopathogenic phenomeon is now being investigated as a routine procedure for the isolation and typing of poliomyelitis strains. The practicability of the test is seen from the fact that virus content of stool specimens can be directly and simply ascertained. Furthermore, the sensitivity of this method has been compared favorably with animal inoculation,[424] and it is possible to isolate and type poliomyelitis strains in 12 days (Youngner and co-workers,[425] Robbins and co-workers,[91] Riordan and co-workers [92]). In May, 1953, Scherer and co-workers [426] reported a decrease in time for obtaining cytopathogenic changes to 24 hours by the use of HeLa malignant cells as a culture substrate for virus. The sensitivity of this technique for the presence of virus is also utilized for epidemiologic purposes by testing serum-virus mixtures to determine the presence or absence of antibodies.

Youngner and his associates [416n] found the Brunhilde strain to be a strongly cytopathogenic reactor. While the Y-SK strain of Lansing is also a strong reactor, other Lansing prototypes have produced few cytologic changes and have required 12 to 15 days for evaluation of virus content in comparison to 6 days for the Brunhilde strain. The replacement of monkey testicular tissue in the cultures by human embryonic skin-muscle tissue reduced this fluctuation. In general agreement with these investigators are the reports of others.[427] It has been suggested [428] that

424. Footnote 416*k*. Footnote 91. Footnote 93.

425. Youngner, J. S.; Lewis, L. J.; Ward, E. N., and Salk, J. E.: Am. J. Hyg. **55**:347, 1952.

426. Scherer, W. F.; Syverton, J. T., and Gey, G. O.: J. Exper. Med. **97**:675, 1953.

427. (*a*) Ledinko, N.; Riordan, J. T., and Melnick, J. L.: Proc. Soc. Exper. Biol. & Med. **78**:83, 1951. (*b*) Footnote 416*k*. (*c*) Footnote 92. (*d*) Footnote 91.

428. Footnote 416*n* and *k*. Footnote 427*a*.

it may be possible to select strongly cytopathogenic representatives from each of the immunologic types for purposes of antibody titration.

The economic and scientific advantages of the cytopathogenic technique are obvious. Unfortunately, so far as is known, the procedure differs in detail from laboratory to laboratory, and no optimum procedure has been established. Undoubtedly proper standards will be made available in time so that the same test conditions prevail in all laboratories.

Prolonged cultivation of the Lansing and Brunhilde strains of virus have revealed no alteration of the cytopathogenic activity (Weller and co-workers [416b]). However, in the case of the Lansing strain, a decrease in mouse virulence was noted without a corresponding decrease in monkey virulence, and, similarly, in the case of the Brunhilde strain there was a decrease in monkey virulence. These observations were made on suspensions of human embryonic skin-muscle tissue in serum ultrafiltrate.

Roller Tube Versus Suspended Cell Cultures: The poliomyelitis virus has been grown without difficulty in both the roller tube and the suspended cell types of tissue

TABLE 2.—*Comparison Between Suspended Cell and Roller Tube Cultures of Poliomyelitis Virus*

Roller Tube	Suspended Cell
Choice for cytopathogenic evaluation; cell changes observed directly	Cellular changes; cytopathogenic phenomenon not as clear as in roller tubes
With small inoculum of virus, growth of virus more rapid and titer higher than in suspended cell culture; choice for direct isolation and typing of strains	With large inoculum of virus, a yield comparable to infected monkey tissue obtained
Small amount of tissue required	Convenient and economical; no equipment necessary
At least one tissue (human uterus) supporting virus growth here; negative in suspended cell	

culture. Because there are certain differences in virus activity in each type of culture (Robbins, Weller, and Enders [28]; Weller, Enders, Robbins, and Stoddard [416b]), a brief comparison based on recent reports is presented in Table 2.

Antibiotics: Antibiotic mixtures are utilized by almost all the laboratories interested in poliomyelitis propagation to prevent contamination of cultures. Most commonly used are mixtures of penicillin and streptomycin, the amounts varying in different reports, usually between 50 units and 250 units per milliliter of each antibiotic. Recommendations for mixtures of 50 units of penicillin and 50 γ of streptomycin per milliliter have been made by Weller and co-workers,[429] Ledinko and co-workers,[416k] and Thicke and co-workers.[58] As much as 5,000 units of penicillin and 5,000 γ of streptomycin have been used by Syverton and Scherer,[41] and they have noted that even with these high concentrations of antibiotics there has been no apparent effect upon the cellular metabolism or upon virus multiplication.

Vaccine from Tissue Culture: Aside from the general academic interest which has stimulated the great effort in propagation of poliomyelitis virus, the possibility of obtaining an effective vaccine, low in protein, from poliomyelitis cultures has been the ultimate goal of most investigators. It is likely that many immunization

429. Footnote 410. Footnote 416*b*.

experiments have been carried out with the tissue culture vaccine and will be reported in the future. Only a few communications in this direction have come to our attention. In the first, Salk and his associates [430] found that tissue culture virus of each of the three antigenic types mixed with mineral oil adjuvants and tested in monkeys compared favorably in antigenic activity with central nervous system virus. Salk [416j] has confirmed this work by studying the antibody potential of formalinized tissue culture virus grown in the presence of human kidney tissue and injected into 90 humans. Similarly, Wood and co-workers,[59] using tissue culture virus grown in synthetic media, also apparently obtained excellent results in monkeys with the three types of virus. Franklin, Duncan, Wood, and Rhodes [57b] have been successful in cultivating the virus in a controlled environment (synthetic medium No. 199) and have noted the possibility of obtaining large amounts of tissue culture virus for vaccine production.

Embryonated Eggs.—Until 1952, the inability of any of the known types or strains of poliomyelitis virus to grow in the developing chick embryo had been considered a characteristic distinguishing these viruses from other neurotropic agents (Casals and Olitsky [431]). Numerous unsuccessful attempts were made to adapt human poliomyelitis viruses to the developing chick embryo (Burnet [432]; Stimpert [433]; Kast and Kolmer [434]; Pollard [435]; Yager, Olitsky, and Lahelle [436]).

In 1952 it was reported that after 119 serial passages in suckling hamsters, the MEFI-A strain of human poliomyelitis virus was maintained for 41 generations in the developing chick embryo by the yolk sac route of inoculation (Roca-Garcia and co-workers [29]). The MEFI strain also propagated following allantoic sac inoculation (Cabasso and co-workers [30]).

Propagation in eggs of a mouse Lansing strain without previous hamster passage was demonstrated when the embryonated eggs were first treated with cortisone and the virus was inoculated into the allantoic cavity (Dunham and Ewing [437]).

PSEUDORABIES VIRUS

Tissue Cultures.—In 1933, Traub [438] reported successful transmission of the virus of pseudorabies through 49 subcultures on chick embryo tissue in Tyrode's solution. Intranuclear inclusion bodies were found in cultured tissues, and the final transfer was infective for mice. The same author later [438b] reported six passages of the virus in cultures of immune guinea pig testis.

Czerey-Pechany, Belady, and Ivanovics [96] cultivated the Ay-XXX strain of pseudorabies in minced chick embryo suspended in Simms' solution-serum ultra-

430. Salk, J. E.; Lewis, L. J.; Bennett, B. L., and Youngner, J. S.: Fed. Proc. **11**:480, 1952.

431. Casals, J., and Olitsky, P. K.: Diagnosis of Viral and Rickettsial Infections, New York, Columbia University Press, 1949, Chap. 6, p. 71.

432. Burnet, F. M.: M. J. Australia **1**:46, 1935.

433. Stimpert, F. D.: Proc. Soc. Exper. Biol. & Med. **41**:483, 1939.

434. Kast, C., and Kolmer, J. A.: J. Infect. Dis. **61**:60, 1937.

435. Pollard, M.: Texas Rep. Biol. & Med. **7**:480, 1949.

436. Yager, R. H.; Olitsky, P. K., and Lahelle, O.: J. Bact. **58**:112, 1949.

437. Dunham, W. B., and Ewing, F. M.: J. Bact. **65**:224, 1953.

438. Traub, E.: (*a*) J. Exper. Med. **58**:663, 1933; (*b*) ibid. **61**:883, 1935.

filtrate for 38 passages with an average medial lethal dose titer of 10^{-5}. These investigators were able to titrate the Ay virus in tissue culture, utilizing its cytopathogenic effects. Such titrations yielded results in close agreement with those obtained from mouse tests. When neutralization tests (Huang, with western equine encephalomyelitis) were performed, it was found that the tissue culture system gave higher neutralization indices than mice. No inclusion bodies were demonstrated in these cultures.

Scherer [260] investigated the relationship between cellular proliferation and virus propagation in cultures of Earle's L-strain cells on glass. In addition to a cell-free control, virus growth was followed in the following three different preparations: in a nutrient fluid of horse serum-embryo extract-Hanks' solution (4:1:5) in which the cells proliferated rapidly, in a fluid medium consisting only of Hanks' solution-serum ultrafiltrate (2:1) in which the cells were less active, and in a maintenance solution (Hanks' solution, with essential amino acids and additional glucose) in which there was no proliferation at 22 to 25 C., although the cells survived for the duration of experimental observations. The pseudorabies virus propagated most readily in the actively proliferating preparations, to a smaller degree in the serum ultrafiltrate solution, and minimally or not at all in the maintenance fluid cultures. Type A inclusion bodies were present but not widespread in the first type of culture, whereas in the latter two types, although cellular destruction was extensive in virus-infected cultures, inclusion bodies were absent.

Embryonated Eggs.—The chorioallantoic membrane and yolk sac [439] were satisfactory sites of inoculation for the virus of pseudorabies (Mesrobeanu [440]; Burnet, Lush, and Jackson [441]; Glover [442]; Bang [443]; El Sabban [439]). The microscopic lesions on the chorioallantoic membrane resembled those of herpes simplex virus.[443] Nuclear inclusions were demonstrated in the yolk sac, and the virus was uniformly lethal for chick embryos.[439] After serial egg passage of the virus, a partial loss of virulence for mice occurred accompanied by an increasing pathogenicity for eggs.[441]

PSITTACOSIS VIRUS

Tissue Cultures.—In 1932, Bedson and Bland [444] first reported cultivation of the virus of psittacosis in hanging drop preparations of mouse serum, salt solution (or chick embryonic extract), and mouse spleen. When normal spleen was used, it was allowed to stand for an hour in the refrigerator in the presence of a fresh psittacosis virus filtrate. Otherwise, infected spleen tissue was used as the explant. After 72 hours of incubation at 37 C., a thousand-fold increase in potency was noted. When the cultured tissues were stained with Giemsa's stain or with a modified Casteneda stain, intracellular inclusions were found.

439. El Sabban, M. S.: Proc. Soc. Exper. Biol. & Med. **71**:423, 1949.

440. Mesrobeanu, I.: Compt. rend. Soc. biol. **127**:1183, 1938.

441. Burnet, F. M.; Lush, D., and Jackson, A. V.: Australian J. Exper. Biol. & M. Sc. **17**:35, 1939.

442. Glover, R. E.: Brit. J. Exper. Path. **20**:150, 1939.

443. Bang, F. B.: J. Exper. Med. **76**:263, 1942.

444. Bedson, S. P., and Bland, J. O.: Brit. J. Exper. Path. **13**:461, 1932.

This work was repeated by Bland and Canti,[446] who used mouse spleen, minced chick embryo, chick embryo lung, leg muscle, and skin. They studied the cycle of development of inclusion bodies and found fibroblasts from leg muscle and cutaneous epithelium best suited for such work. Extracellular formations of inclusions were not seen.

Successful propagation of this virus in chick embryo tissue (Levinthal[446]) and in embryonic chick liver (Haagen and Crodel[447]) has also been reported. During the course of his work with embryonic chick tissue, Levinthal noted a definite predilection of the psittacosis virus for endothelial and epithelial cells.

Yanamura and Meyer[448] demonstrated virus multiplication in suspended cell and agar slant cultures of chick embryo. One strain was carried for 262 passages. These workers showed that saline could not be substituted for Tyrode's solution, but, if Simms' solution was used, the tissue component could be increased two-fold. As long as the tissue fluid ratio (1:60 in Tyrode's solution) was maintained, cultures with fluid depth as great as 4.5 cm. supported virus propagation. The dilution of the infectious material inoculated into tissue cultures determined the incubation period required for attainment of maximum titers. Granulocorpuscles were observed in various cell types (mesenchyme, fibroblast, epithelium, et cetera); on the basis of the presence or absence of these bodies accurate titrations of virus potency were possible. In the two and one-half years the "Maine" strain was sustained in tissue culture, no loss of mouse virulence was noted.

Morgan and Wiseman[449] have used the roller tube and the cellophane techniques to obtain in vitro propagation of psittacosis virus. In the roller tube culture of chick embryo tissue in Simms' solution-serum ultrafiltrate, the fluid contained quantities of elementary bodies. A preliminary formalized vaccine utilizing culture virus was effective.

Embryonated Eggs.—The psittacosis virus propagated on the chorioallantoic membrane producing in one laboratory (Burnet and Rountree[450]) opaque white focal lesions and in another (Lazarus and Meyer[451]) a diffuse gray edematous appearance of the membrane. The yolk sac route of inoculation was also employed (Yanamura and Meyer[448]). Allantoic fluid of intra-allantoically infected eggs yielded the highest concentration of psittacosis virus (Williams,[452] Francis and Gordon[453]). Elementary bodies were observed in the chorioallantoic membrane and allantoic fluid and sparsely in embryo liver and spleen.[454] Mortality of the infected embryo depended on the strain of virus employed (Burnet and Foley,[455] Lazarus and Meyer,[451] Burnet and Rountree[450]). No alteration of mouse virulence could be demonstrated after embryonated egg culture.[451]

445. Bland, J. O., and Canti, R. G.: J. Path. & Bact. **40**:231, 1935.
446. Levinthal, W.: Lancet **1**:1207, 1935.
447. Haagen, E., and Crodel, B.: Zentralbl. Bakt. (Abt. 1) **138**:20, 1937.
448. Yanamura, H. Y., and Meyer, K. F.: J. Infect. Dis. **68**:1, 1941.
449. Morgan, H. R., and Wiseman, R. W.: J. Infect. Dis. **79**:131, 1946.
450. Burnet, F. M., and Rountree, P.: J. Path. & Bact. **40**:471, 1935.
451. Lazarus, A. S., and Meyer, K. F.: J. Bact. **38**:121, 1939.
452. Williams, S. E.: Australian J. Exper. Biol. & M. Sc. **22**:205, 1944.
453. Francis, R. D., and Gordon, F. B.: Proc. Soc. Exper. Biol. & Med. **59**:270, 1945.
454. Williams.[452] Lazarus and Meyer.[451]
455. Burnet, F. M., and Foley, M.: Australian J. Exper. Biol. & M. Sc. **19**:235, 1941.

RABBIT FIBROMA VIRUS

Tissue Cultures.—Faulkner and Andrewes [456] passed an inflammatory strain through 10 culture generations in flask cultures and hanging drop preparations. Rabbit testicular tissue, normal rabbit serum (or plasma), and Tyrode's solution were used. Spleen extract was added to the hanging drops. Virus propagation apparently occurred, since the original titer was 10^{-3} and the original inoculum was diluted 10^{-9} by the eighth subculture. Inclusion bodies were not seen in the cultures.

The same investigators were unable to maintain a fibromatous strain of this virus and noted that the gradual decrease in potency corresponded with degeneration of the cultivated tissue.

Embryonated Eggs.—The tumor-producing strain of rabbit fibroma virus was found to propagate on the chorioallantoic membrane (Smith [457]). Eighteen serial passages on the membrane were carried out, and it was demonstrated that no other route of inoculation gave positive results; further, virus was absent from all but chorioallantoic tissue. There were no specific lesions of the membrane, and egg passage did not alter the nature of lesions in the rabbits.

RABBIT MYXOMA VIRUS

Tissue Cultures.—The virus of rabbit myxoma has been successfully maintained for 20 subcultures in flask preparations containing mononuclear cells (Benjamin and Rivers [458]; Plotz [459]) and for 30 passages in hanging drop cultures of rabbit testicular cells (Haagen [460]). Kidney, lung, white blood cells, and lymph node tissues have also been used with some success.[460] Thompson and Coates,[461] who studied the effect of temperature on the myxoma and other viruses, obtained good results with chick tissue and rabbit serum plus Tyrode's solution.

Actual propagation of the virus was demonstrated by an increase in potency of 100 times after incubation in culture and of 10,000 times after further transfers.[458] Plotz observed an increase of potency of 40,000 times by the 17th subculture, and Haagen observed a dilution activity of 1×10^9. The latter investigator also noted that increase in virulence was much more marked when normal cells were added to the virus-containing tissue. Plotz demonstrated that the virus did not survive more than 14 days without transfer.

Embryonated Eggs.—The virus of rabbit myxoma was cultured on chorioallantoic membranes of the embryonated hens' and ducks' eggs (Lush [462]; Haagen and Du [463]; Hoffstadt and Pilcher [464]; Hoffstadt, Omundson, and Donaldson [465]). Definite opaque raised foci appeared on the membrane three days after inoculation.

456. Faulkner, G. H., and Andrewes, C. H.: Brit. J. Exper. Path. **16**:271, 1935.
457. Smith, M. H. D.: Proc. Soc. Exper. Biol. & Med. **69**:136, 1948.
458. Benjamin, B., and Rivers, T. M.: Proc. Soc. Exper. Biol. & Med. **28**:791, 1930-1931.
459. Plotz, H.: Compt. rend. Soc. biol. **109**:1327, 1932.
460. Haagen, E.: Zentralbl. Bakt. (Abt. 1) **121**:1, 1931.
461. Thompson, R. L., and Coates, M. S.: J. Infect. Dis. **71**:83, 1942.
462. Lush, D.: Australian J. Exper. Biol. and M. Sc. **15**:131, 1937.
463. Haagen, E., and Du, D. H.: Zentralbl. Bakt. (Abt. 1) **143**:23, 1938.
464. Hoffstadt, R. E., and Pilcher, K. S.: (*a*) J. Bact. **35**:353, 1938; (*b*) J. Infect. Dis. **64**:208, 1939.
465. Hoffstadt, R. E.; Omundson, D. V., and Donaldson, P.: J. Infect. Dis. **68**:213, 1941.

No inclusion bodies were observed. Two reports noted no decrease in rabbit virulence after extended culture,[466] but one laboratory claimed loss of virulence for rabbits and a corresponding increase in egg pathogenicity.[463]

RABIES VIRUS

Tissue Cultures.—Successful in vitro cultivation of rabies virus was reported in 1936 by Kanazawa,[467] who employed rabbit embryo brain and Tyrode's solution without serum. Since that date it has been propagated in cultures of mouse or rat embryo brain (Bernkopf and Kligler,[468] Schultz and Williams,[469] Webster and Clow,[470] Kligler and Bernkopf,[471] Parker and Hollender [472]), brains of mice up to 14 days of age,[472] chick embryo brain,[473] and whole chick embryo (Plotz and Reagan [474]). However, Parker and Hollender were unable to cultivate rabies in chick embryo brain cultures, even when the virus had been adapted to chick tissue by serial intracerebral passage in embryonated eggs. It has not been possible to obtain multiplication in cultures using brain tissue from mice more than 14 days of age.[475]

Although Kanazawa [467] claimed to have obtained multiplication in cultures without serum, all other investigators have found serum or serum ultrafiltrate to be essential. Monkey serum has been used in most studies.[476] Kligler and Bernkopf [471] reported that human or monkey serum was essential for growth of the virus; when these were replaced by other sera, the virus failed to grow. Webster and Clow found that horse serum could replace monkey serum, and Parker and Hollender obtained culture titers as high as 10^{-6} when rabbit serum and balanced salt solution (Simms') were used. The latter investigators demonstrated that in some subcultures the substitution of ox serum ultrafiltrate for rabbit serum resulted in a lower titer but that at the end of the series of subcultures the titers were the same.

Plotz and Reagan [474] were the only workers to cultivate the street virus directly from the brain of a human patient with rabies or from the brain of a rabid dog. They carried these strains through 11 and 9 subcultures. All other investigators have used fixed or early animal passage virus. In addition, Plotz and Reagan successfully used minced whole chick embryo instead of brain tissue (embryonic or from mice up to 14 days of age). These workers used the "floating cell" type, which they asserted had the advantage of providing a large mass of proliferating cells considered by them to be essential for a high yield of rabies virus in culture.

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466. Footnote 464 *a*. Footnote 462.

467. Kanazawa, K.: Jap. J. Exper. Med. **14**:519, 1936.

468. Bernkopf, H., and Kligler, I. J.: Brit. J. Exper. Path. **18**:481, 1937; Proc. Soc. Exper. Biol. & Med. **45**:332, 1940.

469. Schultz, E. M., and Williams, G. F.: Proc. Soc. Exper. Biol. Med. **37**:372, 1937.

470. Webster, L. T., and Clow, A. D.: (*a*) Science **84**:487, 1936; (*b*) J. Exper. Med. **66**: 125, 1937.

471. Kligler, I. J., and Bernkopf, H.: Am. J. Hyg. **33**:1, 1941.

472. Parker, R. C., and Hollender, A. J.: Proc. Soc. Exper. Biol. & Med. **60**:94, 1945.

473. Footnote 469. Footnote 470.

474. Plotz, H., and Reagan, R.: Science **95**:102, 1942.

475. Footnote 471. Footnote 472.

476. (*a*) Kligler, I. J., and Bernkopf, H.: Nature, London **143**:899, 1939. (*b*) Footnote 470. (*c*) Footnote 474. (*d*) Footnote 471.

Plotz and Reagan and Parker and Hollender stressed the need for using the ground culture as subculture inoculum and potency test material. Kligler and Bernkopf,[471] using embryonic mouse or rat brain, found that culture virus lost some of its peripheral infectivity for mice (intraperitoneal injection). However, there was no decrease in intracerebral infectivity.

Veeraraghavan [477] claimed to have obtained multiplication of rabies in a cell-free medium containing growth factors. However, Kirk and co-workers,[478] following Veeraraghavan's procedure closely, were unable to repeat his results.

In attempting to use culture virus as an immunizing agent, Webster and Clow [479b] noted that mice receiving one intraperitoneal injection of the material were protected against 100 cerebral doses of street virus. Subcutaneous vaccination was not effective. Dogs were also immunized by use of the culture virus. Kligler and Bernkopf [471] effectively immunized mice against subsequent intraperitoneal but not intracerebral inoculation.

Embryonated Eggs.—The embryonated egg supported rabies virus multiplication following chorioallantoic membrane, intracerebral, or yolk sac inoculation (Kligler and Bernkopf,[476a] Dawson,[479] Sigurdsson,[480] Koprowski and Cox [481]). With street virus, no Negri bodies were observed when the chorioallantoic membrane was the site of infection; whereas, with the intracerebral technique, inclusions were found in neurons and ependymal epithelium.[479b] The virus was distinctly attenuated after egg passage so that virus parenterally injected into rabbits produced no symptoms.[481] A vaccine was prepared and has been used effectively (Koprowski and Black [482]).

RIFT VALLEY FEVER VIRUS

Tissue Cultures.—MacKenzie [483] inoculated material from the heart or liver of an infected mouse into chick embryonic tissue in Tyrode's solution and was successful in transmitting the virus of Rift Valley fever through 13 culture generations without loss of virulence. Dilution of the original inoculum was estimated at $1:1.5 \times 10^{18}$. Saddington [484] repeated this work and reported a series of 12 subcultures, the final culture of which was virulent for mice.

Embryonated Eggs.—Saddington [484] transferred the virus through five eggs and noted constant virulence for the embryos. Inflammatory and necrotic changes were seen in the membranes and in the livers.

ROUS SARCOMA VIRUS

Tissue Cultures.—In 1926, Carrel [485] stated that the Rous sarcoma virus could be grown at will in cultures of chicken leucocytes in small quantities of plasma, Tyrode's solution, and embryonic extract. Subcultures were made every two to

477. Veeraraghavan, N.: Indian J. M. Res. **34**:207, 1946; Nature, London **159**:782, 1947.
478. Kirk, R.; Haseeb, M. A., and Davis, A. T.: J. Trop. Med. **53**:167, 1950.
479. Dawson, J. R.: (*a*) Science **89**:300, 1939; (*b*) Am. J. Path. **17**:177, 1941.
480. Sigurdsson, B.: J. Exper. Med. **78**:341, 1943.
481. Koprowski, H., and Cox, H. R.: Proc. Soc. Exper. Biol. & Med. **68**:612, 1948.
482. Koprowski, H., and Black, J.: Proc. Soc. Exper. Biol. & Med. **80**:410, 1952.
483. MacKenzie, R. D.: J. Path. & Bact. **37**:75, 1933.
484. Saddington, R. S.: Proc. Soc. Exper. Biol. & Med. **31**:693, 1934.
485. Carrel, A.: J. Exper. Med. **43**:647, 1926.

three weeks. When pure strains of normal fibroblasts were grown in the presence of the virus, the cultures did not produce tumors in chickens. This finding was consistant with previous reports by the same author, that filtered extracts of Rous sarcoma deteriorated in the presence of fibroblasts [486] and multiplied readily in the presence of leucocytes.[487]

Contradicting the observations of Carrel is the work of Ludford,[488] who investigated the action of Fujinami sarcoma virus on tissue cultures and experienced little difficulty in growing the virus on fibroblasts. This investigator also reported similar results with a classical strain of Rous sarcoma virus and obtained no growth of the virus in the presence of monocytes. In agreement with Ludford are the results of Halberstaedter and co-workers,[489] who reported growth of the Rous sarcoma agent in fibroblasts from embryonic chick myocardium. More recently Sanford, Likely, Brian, and Earle [24] have reported at length on the question of the cell specificity of the Rous sarcoma virus and the action of this agent in continuous culture. This work seems to have answered several questions and is given in detail here. The following cells were used as substrate for virus; chicken monocytes, fibroblasts, embryonic spleen, and mouse fibroblasts. The general techniques for cultivation of cells developed in Earle's laboratory have been discussed (Introduction), and these procedures have been followed in general in the Rous sarcoma study with three types, i. e., plasma clot, cellophane technique, and glass substrate. Nutritive media varied and under different experimental conditions consisted of horse or chicken serum, chick embryo extract, and Earle's balanced saline. All chickens used in these experiments were tested for naturally occurring antibodies against the test virus. In the case of antibodies that might be present in the chicken plasma, transfer of the cultures to cellophane or glass substrate and the deletion of the plasma nullified that problem. The two other potential sources of naturally occurring sarcoma virus were also tested for a filterable agent, i. e., the 9-day chick embryos used for chick extract, as well as the chicken fibroblasts used as cell substrate. The virus was a recognized strain (Hynson, Westcott, and Dunning) of known potency. When used in the experiments, filtrates of the sarcoma culture were added to the test solutions.

During the course of these experiments the ability of the Rous sarcoma virus to remain active for four days at 37.5 C. in a cell-free preparation containing nutritive material was noted.

In 18 so-called preliminary experiments, the following results were obtained by Sanford and her co-workers. Twelve strains of chicken fibroblasts cultured for 4 to 133 days supported the growth of Rous sarcoma virus, as evident in the production of large saracomatous tumors 7 days after inoculation of test material in 461 of 562 injections. The majority of negative tests occurred in first passage material, and there is little doubt of the actual propagation of the viral agent, since in the positive cultures there had been made approximately 87 fluid renewals and 4 transplantations of the cells.

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486. Carrel, A.: Compt. rend. Soc. biol. **92**:477, 1925.

487. Carrel, A.: Compt. rend. Soc. biol. **91**:1069, 1924.

488. Ludford, R. J.: Am. J. Cancer **31**:414, 1937.

489. Halberstaedter, L.; Doljanski, L., and Tenenbaum, E.: Brit. J. Exper. Path. **22**:179, 1941.

In the case of 13 strains of chicken monocytes cultured for 3 to 29 days, only 18 of the 142 injections of infected cultures or culture fluid produced sarcomas. It is important that 15 of the 18 sarcomas stemmed from injections of material within the first week of virus treatment of the cultures. Furthermore, the three remaining positive injections came from preparations injected in chickens 11 days after virus treatment. When the monocytes plus virus were studied later than 11 days, no sarcoma was produced in 82 injections.

Also tested was the capacity of nonspecific mouse fibroblasts (strain L) to support the Rous sarcoma virus. No evidence of virus activity was found in 11 tests.

In three final experiments involving numerous injections into chickens, numerous fluid renewals, as well as some transplantations of culture components, and observation of some of the cells for as long as five months, the previously noted experiments were confirmed. The results of this report have apparently clarified the in vitro habits of the Rous sarcoma virus. It is to be noted that these workers have had the advantages of newer techniques available for the preparation and culture of cells and of broader experience in cultivation of viruses. Until evidence is available to the contrary, it would seem that the fibroblast of the chicken is the tissue cell of choice for the propagation of the Rous sarcoma virus.

Embryonated Eggs.—The virus of the Rous sarcoma was transmitted through embryonated eggs on the chorioallantois for 30 egg passages, with a titer of 10^{-4} in late passages (Keogh [490]). Small inocula gave rise to small specific lesions on the membrane. When large inocula were used, sarcomatous lesions appeared, and a hemorrhagic disease also resulted.

SANDFLY FEVER VIRUS

Tissue Culture.—Shortt, Pandit, and Rao [491] claimed the cultivation of the sandfly fever virus in suspended cell cultures of minced chick embryo. The presence of virus was evidenced in their opinion by distinctive lesions on the chorioallantoic membranes when 18th-passage material was inoculated into embryonated eggs.

Embryonated Eggs.—Sandfly fever virus was reported by three laboratories to have been cultured on the chorioallantoic membrane of embryonated eggs (Shortt, Pandit, and Rao [491]; Demina and Levitanskaja [492]; Khodukin and co-workers [493]). As evidence of the presence of virus, the authors have cited characteristic changes in the chorioallantoic membrane,[494] the absence of such nonspecific membranal lesions when the virus was neutralized with serums from patients recovered from the disease,[492] and the capacity of virus-infected chorioallantoic membrane material to produce the clinical syndrome of sandfly fever in human patients.[492]

The experimental data in support of sandfly fever virus propagation are tenuous, being based on uncertain histopathologic changes. Until evidence is forthcoming for actual demonstration of a virus, this agent is placed in Group III.

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490. Keogh, E. V.: Brit. J. Exper. Path. **19**:1, 1938.

491. Shortt, H. E.; Pandit, C. G., and Rao, R. S.: Indian J. M. Res. **26**:229, 1938.

492. Demina, N. A., and Levitanskaja, P. B.: Med. Parasitol. & Parasit. Dis. **9**:272, 1940; abstracted Trop. Dis. Bull. **40**:305, 1943. Demina, N. A.: Med. Parasitol. & Parasit. Dis. **10**:271, 1941; abstracted Trop. Dis. Bull. **40**:305, 1943.

493. Khodukin, N. I.; Soshnikova, M. N., and Kevorkova, V. I.: J. Microbiol., Moscow **10**:1154, 1943; abstracted Trop. Dis. Bull. **41**:938, 1944.

494. Footnote 491. Footnote 492.

SERUM HEPATITIS VIRUS

Embryonated Eggs.—The virus of serum hepatitis was carried through 15 passages in the amniotic cavity of the chick embryo (Drake, Henle, Henle, and Stokes [495]). Intramuscular injection of the 9th and 15th passage material produced hepatitis without jaundice in four of six and two of five human volunteers, respectively.

SHEEP POX VIRUS

Tissue Cultures.—Bridre [496] inoculated flasks containing sheep testicular cells, serum, and Drew's solution with fresh infectious material. The fourth subculture was active at a dilution of 1:10,000 and produced typical lesions in sheep. No growth of the virus occurred after 14 days.

In view of the small number of subcultures, the quick loss of virus, and the lack of confirmation, the propagation of sheep pox virus in tissue culture should be regarded as questionable.

Embryonated Eggs.—Cultivation of the sheep pox virus on the chorioallantoic membrane was reported (Gins and Kunert,[497] Rao [498]). The infection resembled that of vaccinia.

TESCHEN DISEASE VIRUS

Tissue Culture.—Horstmann,[499] in a comprehensive study of Teschen disease, attempted to demonstrate virus multiplication in tissue culture. Minced chick embryo in Simms' solution-serum ultrafiltrate inoculated with Detusici strain virus was incubated for 16 to 20 days at 36 C., with fluid changes every 4 to 5 days; three culture passages were carried out. First, second, and third passage material were each tested in two pigs. One pig of the two given injections of material from the first culture generation exhibited encephalomyelitic symptoms. The pigs treated with later passages showed minor histological changes suggestive of a specific diagnosis, but no symptoms were noted. One experiment with the Reporje virus strain proved negative. Horstmann concluded that survival or slight multiplication of the virus for 17 days may have occurred in tissue culture. She also presents for consideration the possibility that the change in virus activity in the second and third passages may have represented adaptation rather than negative results.

More extensive tissue culture studies utilizing a broader spectrum of tissue, media, and subculture factors may give more positive findings.

TRACHOMA VIRUS

Tissue Culture.—In 1937, Harrison and Julianelle [500] reported uniformly negative results in an impressive study of the virus of trachoma which included many types of tissue cultures. Inoculums, explanted tissues, and mediums were varied in more than 1,100 tissue cultures, but according to the authors the virus did not even survive, although its presence before inoculation had been frequently proved

495. Drake, M. E.; Henle, W.; Henle, G., and Stokes, J.: Fed. Proc. **11**:467, 1952.
496. Bridre, J.: Compt. rend. Soc. biol. **119**:502, 1935.
497. Gins, H. A., and Kunert, H.: Deutsche tierärztl. Wchnschr. **45**:257, 1937.
498. Rao, R. S.: Indian J. M. Res. **26**:497, 1938.
499. Horstmann, D. M.: J. Immunol. **69**:379, 1952.
500. Harrison, R. W., and Julianelle, L. A.: Am. J. Ophth. **20**:360, 1937.

by animal infection. No inclusion bodies were found. Similarly, Busacca [501] and Thygeson [276] were unable to demonstrate propagation of the trachoma virus.

Poleff,[502] however, asserted that he was successful in passing the virus through five subcultures in a modified Maitland medium described by Nigg and Landsteiner.[503] He also maintained that within two to three days there was a characteristic membrane formation of cells in culture and that inclusion bodies were present and transmissible to the rabbit by intraocular inoculation. A rabbit infected in this manner was used as a further source of infection for two other animals. No mention was made of increase in potency.

In view of the negative results reported by the majority of workers and the small number of passages noted in the single claim to success, trachoma virus should be considered as not having been grown in vitro.

Embryonated Eggs.—When human conjunctival tissue infected in vivo with trachoma virus was grafted onto the chorioallantoic membrane of embryonated eggs, cell proliferation without evidence of virus multiplication was observed (Julianelle [504]). One worker claimed positive propagation of the virus in the yolk sac for at least nine passages (Machiavello [505]). Another laboratory reported a series of experiments in which the virus was alternately inoculated into monkeys and into the yolk sac of the embryonated egg (Stewart and Badir [506]). In this study, monkeys infected with material from the third egg transfer exhibited typical trachomatous follicles.

VARICELLA VIRUS

Tissue Cultures.—Weller and Stoddard [507] reported the presence of inclusion bodies in suspended cell cultures of human embryonic tissue inoculated with virus from vesicles of infected children. The tissue was incubated three to five days prior to the addition of virus. After 10 to 20 days of incubation at 35 C., with fluid changes every three to five days, passages were attempted. No inclusion bodies were demonstrable in the subculture, but it was noted that this absence of inclusion bodies did not rule out the presence of propagating virus.

Unfortunately; an experimental host for the demonstration of varicella is not available. While it is possible that the report by Weller and Stoddard demonstrates propagation of the virus, the lack of a host and the loss of inclusion bodies in subcultures require the placing of this agent in Group II, pending additional work.

Embryonated Eggs.—Varicella virus cultivation in the embryonated egg has been unsuccessful (Beveridge and Burnet [5]).

VARIOLA AND VACCINIA VIRUSES

Tissue Cultures.—In 1906, Aldershoff and Boers [1] reported successful maintenance of vaccinia virus outside the living body. Their experiments were extended

501. Busacca, A.: Arch. opht. **2**:116, 1938.
502. Poleff, L.: Presse méd. **45**:800, 1937.
503. Nigg, C., and Landsteiner, K.: J. Exper. Med. **55**:563, 1932.
504. Julianelle, L. A.: Am. J. Ophth. **26**:280, 1943.
505. Machiavello, A.: Rev. ecuator. hig. (No. 2) **1**:1, 1944; abstracted, Trop. Dis. Bull. **45**:1112, 1948.
506. Stewart, F. H., and Badir, G.: J. Path. & Bact. **62**:457, 1950.
507. Weller, T. H., and Stoddard, M. B.: J. Immunol. **68**:311, 1952.

by Steinhardt, Israeli, and Lambert [2] in 1913 and by Steinhardt and Lambert [508] in 1914, who noted an increase in potency of the virus from 6 to 10 times.

Since these first successful experiments, numerous investigators have reported propagation of vaccinia virus in tissue cultures of all types. The virus appears to grow well in the presence of many tissues, and good results have been obtained with in vitro preparations containing rabbit and guinea pig testicle,[509] corneal tissue,[510] rabbit kidney,[511] chicken kidney,[512] embryonic chick,[513] Kupffer cells,[514] chick embryo heart,[513e] fibroblasts from rabbit and chick embryo cardiac muscle or from rabbit testicle,[515] mouse embryo,[513d] and rabbit leucocytes.[516a]

Prior to 1939, the apparent predilection of vaccinia virus for epithelial tissue was stressed.[516] Haagen [517] had gone a step farther in maintaining that vaccinia virus could be cultivated for only a few generations if fibroblastic tissue predominated, and he concluded that epithelial and endothelial cells were necessary for growth of the virus.

Since that time it has been conclusively demonstrated that fibroblastic tissue from rabbit cardiac muscle,[515a] from chick embryo cardiac muscle,[513e] or from rabbit testicle [515a] will effectively support the multiplication of vaccinia virus. Further, the virus multiplies in chick embryo cardiac muscle tissue (which is of mesodermal and entodermal origin),[513e] and in Kupffer cells [514] and in rabbit leucocytes.[516a] It is interesting that when vaccinia was propagated in the rabbit fetus in utero, virus was demonstrated in all tissues tested and could be recovered from liver, lungs, brain, skin, placenta, and kidney.[518] Thus, vaccinia virus apparently has a much broader tissue affinity than was previously appreciated. The virus can be cultivated at will with routine titers of 10^{-4} to 10^{-6}.

Propagation of the virus has been shown to occur in vitro when the cells were viable but not proliferating (Rivers, Haagen, and Muckenfuss [512a]; Plotz [519]) and

508. Steinhardt, E., and Lambert, R. A.: J. Infect. Dis. **14**:87, 1914.

509. (*a*) Parker, F., and Nye, R. N.: Am. J. Path. **1**:325, 1925. (*b*) Plotz, H.: Compt. rend. Acad. sc. **174**:1265, 1922. (*c*) Mervin, S., and Schmerling, A.: Zentralbl. Bakt. (Abt. 1) **100**: 310, 1926. (*d*) Harde, E. S.: Compt. rend. Soc. biol. **78**:545, 1915.

510. (*a*) Cracium, E. C., and Oppenheimer, E. H.: Bull. Johns Hopkins Hosp. **37**:428, 1925; (*b*) J. Exper. Med. **43**:815, 1926. (*c*) Rivers, T. M.; Haagen, E., and Muckenfuss, R. S.: ibid. **50**:665, 1929.

511. (*a*) Muckenfuss, R. S., and Rivers, T. M.: J. Exper. Med. **51**:149, 1930. (*b*) Haagen, E.: Zentralbl. Bkt. (Abt. 1) **120**:304, 1931. (*c*) Footnote 10.

512. (*a*) Rivers, T. M.; Haagen, E., and Muckenfuss, R. S.: J. Exper. Med. **50**:181, 1929. (*b*) Maitland and Maitland.[8]

513. (*a*) Rivers, T. M.: J. Exper. Med. **54**:453, 1931. (*b*) Carrel, A., and Rivers, T. M.: Compt. rend. Soc. biol. **96**:848, 1927. (*c*) Kurotchkin, T. J.: Proc. Soc. Exper. Biol. & Med. **41**:407, 1939. (*d*) Thompson, R. L.; Price, M. L.; Minton, S. A.; Elion, G. B., and Hitchings, G. H.: J. Immunol. **65**:529, 1950. (*e*) Feller, A. E.; Enders, J. F., and Weller, T. H.: J. Exper. Med. **72**:367, 1940. (*f*) Footnote 42. (*g*) Footnote 510 *c*. (*h*) Footnote 101. (*i*) Footnote 107. (*j*) Footnote 102.

514. Beard, J. W., and Rous, P.: J. Exper. Med. **67**:883, 1938.

515. (*a*) Florman, A. L., and Enders, J. F.: J. Immunol. **43**:159, 1942. (*b*) Footnote 513 *e*.

516. Nauck, E. G., and Robinow, C.: Zentralbl. Bakt. (Abt. 1) **135**:437, 1935. Footnote 510 *a*.

517. Haagen, E.: Zentralbl. Bakt. (Abt. 1) **109**:31, 1928.

518. Gallagher, F. W., and Woolpert, O. C.: J. Exper. Med. **72**:99, 1940.

519. Plotz, H.: Les ultravirus des maladies humaines, Paris, Norbert Maloine, 1938.

in the presence of living, actively proliferating cells (Cracium and Oppenheimer [510]; Steinhardt, Israeli, and Lambert [2]; Williams and Flournoy [520]; Hirano [521]). The extensive tolerance of the virus of vaccinia for variations of cellular metabolism is emphasized by the important contribution of Li and Rivers,[42] who succeeded in growing the virus in a preparation of chick embryo tissue and Tyrode's solution. The cultivation of a virus was thus reduced to a matter of the simple in vitro preparations (living cells plus a balanced salt solution). This work was later confirmed by Rivers,[513a] Rivers and Ward,[522] and Plotz,[519] who respectively succeeded in passing vaccinia strains through 15, 90, and 150 culture passages.

Maitland and Laing [10] further simplified the culture by growing vaccinia virus for several culture generations in minced kidney suspended in 0.85% NaCl. There was no significant difference between the titer of cultures in saline and of parallel cultures in Tyrode's solution.

The growth requirements of vaccinia virus were further studied by Maitland, Laing, and Lyth,[523] who found a correlation between respiratory activity of tissues and their capacity to act as a substrate for virus growth. On this basis, testis, kidney, liver, and spleen could be employed in descending order for vaccinia cultures. Thompson [101] agrees in principle with these investigators and on the basis of extensive studies has stated that any agent which decreases oxygen tension of the medium or decreases oxygen uptake by the tissue will depress the development of vaccinia virus. The same author,[107] in another study, demonstrated that depth of medium was unimportant provided care was taken to maintain the tissues without crowding. It was, therefore, possible for Thompson to cultivate vaccinia in preparations where the columns of fluid were as great as 115 mm. A brief summary of Thompson's extensive studies concerned with the growth requirements of vaccinia has been presented in the Introduction.

Agreement has been general that in the case of vaccinia the presence of actively proliferating cells is conducive to good yields of virus. Feller and co-workers [513e] studied the growth curves of vaccinia virus with chick embryo cardiac muscle or whole minced chick embryo in roller tubes. With the former cell, a maximum titer was reached on the 5th day, compared to a maximum on the 24th day with the whole chick tissue. In both cases, however, maximum titers were maintained for the duration of the experiment, i. e., 59 days. Maitland and Laing,[10] using large amounts of minced rabbit kidney in Tyrode's solution (a ratio of tissue to fluid of 1 : 25), obtained maximum titers of between 10^{-4} to 10^{-5} on the fourth to seventh days. When they increased the ratio of tissue to fluid to 1 : 400, the lag period was extended several days.

Confirmation of the presence of inclusion bodies in in vitro cultivation of vaccinia has not been lacking. In addition to reports by earlier workers (Aldershoff and Boers,[1] Parker and Nye [509a]), Rivers, Haagen, and Muckenfuss [510c]; Rivers and Ward,[522] and Haagen [511b] reported the presence of these bodies in cultures. According to Plotz,[519] plasma and proliferating cells were important factors in the production of inclusion bodies. In recent studies [513e] inclusions have been observed in fibroblast tissue cultures.

520. Williams, A. W., and Flournoy, T., in Studies from the Rockefeller Institute for Medical Research, New York, Rockefeller Institute for Medical Research, 1905, Vol. 3.

521. Hirano, N.: Am. J. Path. **1**:635, 1925.

522. Rivers, T. M., and Ward, S. M.: J. Exper. Med. **58**:635, 1933.

523. Maitland, H. B.; Laing, A. W., and Lyth, R.: Brit. J. Exper. Path. **13**:90, 1932.

Investigations of immune reactions in vaccinia tissue cultures have yielded somewhat conflicting results. Preparations containing plasma and corneal tissue of immune animals or normal tissues to which immune serum had been added did not support growth of the virus (Steinhardt and Lambert,[508] Nye and Parker [524]). However, according to Rivers and Ward, [510c] vaccinia virus survived at least 48 hours in cultures of immune cornea in normal plasma and to a less extent in cultures of immune cornea in immune serum. When normal cornea was inoculated in vitro with virus and then cultivated in antivaccinia plasma, typical lesions with Guarnieri bodies occurred in 24 to 48 hours. If, however, immune cornea was used in normal or antivaccinia plasma, a mild reaction or no reaction at all occurred. As a result of these findings, Rivers and Ward hypothesized that, once the virus had combined with the cell, it could not be neutralized by addition of immune substances. This was partially borne out by Rivers, Haagen, and Muckenfuss,[233] who observed that immune corneas inoculated and cultivated in immune plasma showed very mild infection with only occasional Guarnieri bodies.

Florman and Enders [515a] investigated the role of the phagocytic cell in vaccinia cultures. In previous studies with phagocytes, conflicting results had been reported in cultures maintained for only short periods of time. Florman and Enders, however, used the roller tube technique to maintain actively proliferating rabbit monocytes for long intervals of time, with the virus propagating for at least three weeks. Although the phagocytic cell exerted a temporary inhibiting effect upon the virus, it was an unusually sensitive indicator for the presence of virus, as demonstrated by its capacity to act as a host when exposed to apparently noninfectious mixtures of immune serum and vaccinia virus. It is emphasized by the authors that this property of the monocyte may be a mechanism for dissemination of virus or for maintenance of latent infections in the natural host.

There appear to be certain similarities between the monocytic culture work and the studies of Beard and Rous,[514] who investigated the action of vaccinia and Kupffer cells both in vitro and in vivo. They noted that the activity of vaccinia virus was reduced when a mixture of the virus and Kupffer cells was allowed to stand for a few minutes before intradermal injection. This apparently confirmed Ledingham's observation that in skin into which India ink had been injected there was no reaction to the virus owing, he thought, to mobilization of Kupffer cells in response to the ink. However, when Beard and Rous cultivated the virus of vaccinia with Kupffer cells in vitro, the virus proliferated very well during the first six or seven days. Perhaps, as was suggested by the author, a factor present under in vivo conditions contributed to Kupffer cells to create an antivaccinia environment.

The logical outgrowth of in vitro cultivation of the virus of vaccinia was its use in Jennerian prophylaxis. Rivers,[513a] Herzberg,[525] Rivers and Ward,[522] Plotz and Martin,[526] and Lehmann,[527] have studied the possible use of the tissue culture virus as a substitute for calf lymph. In general, the impressions of these workers is that the cultivated virus tends to become attenuated and, at best, can only be used as an adjunct to lymph vaccine. For example, Rivers and Ward [522] reported a decrease

524. Nye, R. N., and Parker, F.: Am. J. Path. **5**:147, 1927.
525. Herzberg, K.: Klin. Wchnschr. **11**:2064, 1932.
526. Plotz, H., and Martin, R.: Bull. Acad. méd., Paris **116**:454, 1936.
527. Lehmann, W.: Med. Klin. **37**:1033, 1941.

in titer of vaccinia cultures from 10^{-6} in the 19th culture generation to 10^{-1} in the 86th generation. Potency was easily reestablished to 10^{-6} by three intratesticular rabbit inoculations, and this "revived" virus retained the titer of 10^{-6} for 60 generations without further animal passage. However, Rivers, Ward, and Baird [528] reported that even this strain was not completely protective, and revaccination within six months to one year with potent calf lymph virus was necessary to induce the desired protection.

Embryonated Eggs.—Since vaccinia was first propagated on the chorioallantoic membrane in 1932 (Goodpasture, Woodruff, and Buddingh [529]) the virus has also been grown at will in the allantoic cavity and amniotic sac (Beveridge and Burnet [5]). The dermal and neurotropic strains produce somewhat different lesions (Keogh [530]), but they can be cultivated with comparative ease (Goodpasture and co-workers [531]). According to Beveridge and Burnet,[5] who summarized this subject in 1946, the early gross changes produced by the two strains appear as small milky opacities on the membrane. With the dermal strain the first change is apparent in 24 hours. At 48 hours the lesions are enlarged (1 to 2 mm. in diameter) and discrete, with an occasional central depression. At 72 hours the lesions are still larger, and new ones appear. In the case of the neurotropic strain, it is 48 hours before the first gross changes are visible. With time, necrosis and hemorrhage and also conspicuous depressions occur in the central portion of the lesions. When the inoculum is massive, coalescent lesions occur which reveal the characteristic pathologic processes of the individual strains. In general, the lesion caused by the dermal strain tends to be proliferative, while that caused by the neurotropic strain is characterized by hemorrhage, thrombosis of vessels, edema, and exudation. Fowl red blood cell agglutinin was demonstrated in egg preparations (Nagler [532]).

Although it was stated that vaccinial strains were not very virulent for the embryo, death, when it did occur, usually took place after the fourth day and was more likely to be produced by the neurotropic strain (Beveridge and Burnet [5]). One laboratory reported that emybryonic involvement by this virus had never been observed (Stevenson and Butler [533]). Guarnieri bodies have been noted by numerous workers (Goodpasture and co-workers,[529] Herzberg [534]).

In the opinion of Goodpasture and his collaborators,[531] a vaccine prepared from the chorioallantoic membrane is preferable to tissue culture vaccine because of its ability to maintain high titers without animal passage and because it appears to have less pathogenicity for man while at the same time conferring a strong degree of immunity.

It is not certain if the potency of vaccinia virus is decreased after long passage through eggs. Kunert [535] found a marked decrease in potency after 50 egg passages.

528. Rivers, T. M.; Ward, S. M., and Baird, R. D.: J. Exper. Med. **69**:857, 1939.
529. Goodpasture, E. W.; Woodruff, A. M., and Buddingh, G. J.: Am. J. Path. **8**:271, 1932.
530. Keogh, E. V.: J. Path. & Bact. **43**:441, 1936.
531. Goodpasture, E. W.; Buddingh, G. J.; Richardson, L., and Anderson, K.: Am. J. Hyg. **21**:319, 1935.
532. Nagler, F. P. O.: M. J. Australia **1**:281, 1942.
533. Stevenson, W. D. H., and Butler, G. G.: Lancet **2**:228, 1933.
534. Herzberg, K.: Zentralbl. Bakt. (Abt. 1) **136**:257, 1936.
535. Kunert, H.: Ztschr. Hyg. **117**:216, 1935.

Lehmann,[536] however, reported that virulence for rabbits increased with egg passage. In a general discussion of the problem, Beveridge and Burnet[5] have presented additional evidence in favor of a vaccine derived from eggs but have also suggested that any possible "alteration of the virus and diminished immunizing power, can be eliminated by using material not more than a few passages from a standard strain of calf lymph."

Variola was shown to grow readily on the chorioallantoic membrane (Torres and de Castro Teixeira[537]; Lazarus, Eddie, and Meyer[538]; Buddingh[539]; Nagler[532]), and diagnoses differentiating variola and varicella (Markham and Bozalis[540]) and variola and vaccinia (Downie and Dumbell[541]) were achieved by egg inoculation. In the latter instance the difference was histopathologic.

VESICULAR STOMATITIS VIRUS

Tissue Culture.—Carrel, Olitsky, and Long[542] inoculated the virus of vesicular stomatitis into flasks containing guinea pig embryonic tissue or bone marrow cells, plasma, Tyrode's solution, and guinea pig or chick extract. When this preparation was incubated for 7 to 10 days and titrated by guinea pig inoculation, an increase in potency of 10 to 1,000 times for each passage was noted. However, not each subculture was infective.

Cox, Syverton, and Olitsky,[543] using filtrates of mouse brains infected with New Jersey and Indiana strains, cultivated these strains in preparations containing Tyrode's solution and minced chick embryos from which the heads and limbs had been removed. Fifteen culture generations for each strain were reported with an increase in potency of 10^6. Later the same authors[544] repeated this work and obtained 35 and 38 culture generations with respective increases in potency of 10^{36} and 10^{91}.

Embryonated Eggs.—The vesicular stomatitis virus was cultivated on the chorioallantoic membrane (Burnet and Galloway,[545] Sigurdsson,[546] Eichhorn and Manthei[547]). Lesions characterized histologically by ectodermal proliferation and ultimate necrosis were apparent on the membrane. Mortality of the embryos depended on their age at the time of inoculation and the strain of virus employed.[546] Virus was present in the skin and liver of the embryo, as well as in the membrane.[545] The egg proved a very sensitive system for virus detection and was satisfactory for testing immune serum.[545]

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536. Lehmann, W.: Zentralbl. Bakt. (Abt. 1) **132**:447, 1934.
537. Torres, C. M., and de Castro Teixeira, J.: Compt. rend. Soc. biol. **118**:1023, 1935.
538. Lazarus, A. S.; Eddie, B., and Meyer, K. F.: Proc. Soc. Exper. Biol. & Med. **36**:7. 1937.
539. Buddingh, G. J.: Am. J. Hyg. **28**:130, 1938.
540. Markham, F. S., and Bozalis, G. S.: J. Bact. **37**:656, 1939.
541. Downie, A. W., and Dumbell, K. R.: J. Path. & Bact. **59**:189, 1947.
542. Carrel, A.; Olitsky, P. K., and Long, P. H.: Compt. rend. Soc. biol. **98**:827, 1928.
543. Cox, H. R.; Syverton, J. T., and Olitsky, P. K.: Proc. Soc. Exper. Biol. & Med. **30**: 896, 1933.
544. Olitsky, P. K.; Cox, H. R., and Syverton, J. T.: J. Exper. Med. **59**:159, 1934.
545. Burnet, F. M., and Galloway, I. A.: Brit. J. Exper. Path. **15**:105, 1934.
546. Sigurdsson, B.: J. Exper. Med. **78**:17, 1943.
547. Eichhorn, E. A., and Manthei, C. A.: J. Am. Vet. M. A. **94**:608, 1939.

VIRUS III

Tissue Cultures.—Andrewes [87] reported in 1929 that he was able to cultivate virus III with comparative ease in Carrel flasks which contained normal and infected rabbit testicular cells, Tyrode's solution, and rabbit plasma or serum. This series was passed through 10 generations with an increased potency of 3.2×10^{9}. He found inclusion bodies occurring regularly in healthy cells. In another series,[548] Andrewes succeeded in carrying the virus through 23 subcultures with an increased potency of at least 8×10^{27}, but he was unable to demonstrate inclusion bodies when the testicular cells were replaced by liver, spleen, kidney, or bone marrow cells.

Topacio and Hyde,[549] with a preparation similar to that used by Andrewes, passed the virus through eight subcultures and demonstrated a final dilution activity of 1:300,000,000. Ivanovics and Hyde [26] repeated the work of previous investigators and obtained propagation of the virus in rabbit tissues other than testis, although the latter was the most receptive for virus III. Pearce [550] passed virus III through 40 consecutive subcultures of minced rabbit testicle, diluting the original inoculum to 10^{-38}, without any apparent decrease in virulence for rabbits. Guinea pig and rat testicular tissue failed to support virus III growth, but serum from resistant species (rat, mouse, guinea pig, horse, cow, goat, and human) did not affect virus propagation in rabbit tissue cultures.

Embryonated Eggs.—One unsuccessful attempt to cultivate virus III in the embryonated egg has been reported (Myers and Chapman [551]).

YELLOW FEVER VIRUS

Tissue Culture.—In the approximately 40-year period during which viruses have been cultivated outside the intact host, yellow fever studies remain classical examples of the usefulness of the in vitro technique. At the same time, reexamination of the problem during recent years has revealed the necessity for studying the cultural requirements of a virus as part of an orderly and detailed program.

The first report of the successful propagation of the yellow fever virus in tissue culture was made by Haagen and Theiler.[552] Various types of tissues were studied: minced kidney and testicular tissue of guinea pigs and rabbits, as well as minced 8 to 10-day chick embryos. The minced embryos were found to be most uniformly satisfactory and were used as routine tissue. In the early studies the supernatant fluid was transferred for subculture, and even in the first report there was a definite increase in potency of the virus in tissue culture, since the 20th subculture was as infectious as the first, and the dilution factor for the virus was estimated at 5×10^{15}. These authors, using the same type of culture in Tyrode's solution and monkey serum, continued the growth of the yellow fever virus for 60 culture generations over a period of eight months. (It should be noted that initiation of the culture was possible because the virus had been modified by intracerebral passage through many mouse generations.)

548. Andrewes, C. H.: Brit. J. Exper. Path. **10**:273, 1929.

549. Topacio, T., and Hyde, R. R.: Am. J. Hyg. **15**:99, 1932.

550. Pearce, J. M.: J. Immunol. **38**:9, 1940.

551. Myers, R. M., and Chapman, M. J.: Am. J. Hyg. **25**:16, 1939.

552. Haagen, E., and Theiler, M.: Zentralbl. Bakt. (Abt. 1) **125**:145, 1932; Proc. Soc. Exper. Biol. & Med. **29**:435, 1932.

Later, Haagen [553] showed that living cells were necessary for growth of the virus and claimed [554] that epithelial cells were best suited to yellow fever propagation. In another series he was able to pass the virus through 100 subcultures and to demonstrate its close affinity for the cells in culture as compared with the fluid components. During these experiments Haagen [50] noted that practically no propagation of the virus occurred if the serum was removed from the culture preparations. In view of later findings which showed that serum was not necessary for yellow fever growth (Findlay and MacCallum,[51] Fox [52]), this report was unfortunate.

Lloyd, Theiler, and Ricci [555] cultivated the pantropic strain and two types of the neurotropic strain in flasks containing serum and Tyrode's solution with either chick or mouse embryonic tissue or adult mouse or guinea pig testicular tissue. The pantropic strain was passed through more than 150 subcultures without intercurrent animal passage over a period of 21 months. Prolonged cultivation of this pathogenic strain caused a consistent progressive inability to produce yellow fever. Virulence for monkeys was regained only after 30 animal passages.

Most important to the development of vaccine were the results of Theiler and Smith,[556] who found that in the presence of chick embryo tissue there was marked loss of both neurotropism and viscerotropism, without loss of antigenicity. According to the same authors a high concentration of virus was best insured by infecting whole chick embryos. Using a vaccine prepared from such infected whole chick embryo, they were able to produce immunity in monkeys as measured by the titers of antibodies that had developed. These titers had no relation to the amounts of virus inoculated. The authors were also able to increase the antibody content in the blood of immune persons by subcutaneous inoculation of the culture virus.

Because of some confusion regarding the labeling of the culture strains, a clarification is given here. In the hands of Theiler and Smith, as has been noted, the Asibi strain lost its virulence for humans and monkeys upon prolonged cultivation, and it was labeled "17D." According to Koprowski and Lennette,[557] the term "17DD" is used for tissue cultures derived from the 195th subculture of the original 17D. When 17DD has been maintained for more than 110 culture generations (i. e., at least three hundred five 17D subcultures), the designation of 17DD-high is followed.

The results obtained by the use of the yellow fever vaccine prepared from tissue cultures of yellow fever virus represent at the present writing the best application of in vitro methods to practical purposes. The vaccine has proved efficacious and convenient. The extent of its success is revealed by the following statistics in the 1937 annual report of the Rockefeller Foundation.[558]

Between February, 1937, and February, 1938, more than 59,000 persons were vaccinated in Brazil. Mild reaction or no reactions at all occurred. More than 95% of the subjects vaccinated in field work showed immunity. In a series of 38,000

553. Haagen, E.: Zentralbl. Bakt. (Abt. 1) **128**:13, 1933. Footnote 50.

554. Haagen, E.: Zentralbl. Bakt. (Abt. 1) **129**:237, 1933.

555. Lloyd, W.; Theiler, M., and Ricci, N. I.: Tr. Roy. Soc. Trop. Med. & Hyg. **29**:481, 1936.

556. Theiler, M., and Smith, H. H.: J. Exper. Med. **65**:767, 1937.

557. Koprowski, H., and Lennette, E. H.: Am. J. Hyg. **40**:1, 1944.

558. Annual Report of the International Health Division of the Rockefeller Foundation for 1937, New York, Rockefeller Foundation, 1938.

vaccinated persons, 69 missed a day or more of work. Not a single serious reaction was recorded for this series of vaccination. In 1938 (January to October), about 800,000 persons were vaccinated in Rio de Janeiro, and no serious complications occurred. In 1944, Bugher and Gast-Galvis [559] confirmed these findings by comparing yellow fever incidence in 600,000 vaccinated persons in Colombia (1 doubtful case) with a nonvaccinated population (243 cases).

Although successful attenuation of the yellow fever virus was obtained in 1932 and the mass vaccination program was a reality by 1937, it was not until 1947 that a disciplined study of the cultural requirements of yellow fever virus was reported by Fox.[52] The method of study and the results in this report are considered sufficiently important to be briefly summarized. It is hoped that the original paper will be consulted as a pattern for study of a virus by in vitro methods. The observations of Fox are based on the cultivation of yellow fever virus in cultures of embryonic chick tissue, chiefly of the suspended tissue type. The following points have been clarified:

1. There is a greater concentration of yellow fever virus in the tissue; approximately 100 times more virus is found in the tissue than in the supernatant fluid.

2. The virus of yellow fever in tissue culture produces little effect upon the tissue itself, the cells remaining capable of propagating virus for at least 40 days after they have been used for virus cultivation of one strain of yellow fever.

3. A direct relationship exists between the factors which stimulate tissue and the amount of virus growth. Thus, embryonic extract which stimulates tissue proliferation was found to increase the amount of virus in the cultures. This was true only within a given range; 15% embryo extract was found to be optimal for the growth of virus. In the same fashion, tissue-inhibiting factors reduced the amount of virus. Consistent with these findings, it was shown that such factors as a great increase in serum concentration curtailed the virus yield. Also, substituting a more actively proliferating tissue for a slower growing tissue (for example, muscle for brain) produced a greater yield of yellow fever virus.

4. The most active or ascending phase of the growth curve of the virus coincides with signs of visible tissue proliferation, which in turn are correlated with changes in pH.

5. Varying the temperature from 39.5 to 27 C. produces different types of growth curves. The curves vary not in form but in time of optimal virus growth. Thus, at 27 C. the peak was delayed until the eighth day. This lag is probably the result of tissue activity. Under such circumstances, however, the slope of the curve after the peak is more gradual, and this is presumably a reflection of the greater stability of the virus at the lower temperature. The maximum titers were obtained at temperatures 32.5 to 35 C.

6. Varying the availability of oxygen in different fashion has little effect upon the virus multiplication. Lowering the oxygen tension to near anaerobiasis appears to stabilize the virus, but at that point multiplication is interfered with.

7. Storing at 5 C. allows preparation of cultures at least 11 days prior to inoculation with virus without impairment of their usefulness for virus cultivation.

8. Serum is not essential for propagation of the yellow fever virus.

559. Bugher, J. C., and Gast-Galvis, A.: Am. J. Hyg. **39**:58, 1944.

Embryonated Eggs.—The virus of yellow fever was transmitted through chorioallantoic preparations for a short number of passages by Elmendorf and Smith [560] and Jadin.[561] Increased potency was apparent in the infectivity of high dilutions of either the chorioallantois or the embryo. No variation in the character of the virus was noted. However, in a later study Penna and Moussatche [562] noted alteration in the yellow fever virus after 39 continuous egg passages, so that the virulent Asibi strain produced no infection in monkeys. Theiler and Smith [563] found that high concentrations of the virus occurred when whole chick embryos were infected. They used this information [563] to prepare a vaccine by cultivating the attenuated 17D strain in embryonated eggs (Theiler and Smith [563]; Smith, Penna, and Paoliello [564]).

In 1947, Fox and Laemmert [565] reviewed and extended the subject of yellow fever cultivation in embryonated eggs for the purpose of improving vaccine production. They obtained data regarding persistence of virus in given tissues, the role of temperature of incubation, and the effects of such factors as the size and preferential route of virus inoculum, age of eggs, and duration of incubation prior to harvesting virus. One of the significant findings in this comprehensive paper was the fact that continued egg passage did not increase the neurotropic virulence of the 17D strain for monkeys or man.

"ANOPHELES A AND B," BUNYAMWERA, BWAMBA, HAEMAGOGUS A AND B, LEUCOCELAENUS, NTAYA, SABETHES, SEMLIKI FOREST FEVER, UGANDA S, "WYEOMYIA," AND ZIKA VIRUSES

Tissue Cultures.—Roca-Garcia [566] maintained the "Anopheles A and B" viruses for 10 serial passages in tissue culture. The preparations consisted of minced mouse embryo suspended in 10% human serum-Tyrode's solution. Subcultures were made every fourth day. The viruses had been isolated in mice from east Colombian mosquitos, and infected mouse brain constituted the original inoculum. No loss in pathogenicity of the virus for mice was observed.

Semliki Forest fever virus was shown by Ginder and Friedewald [567] to propagate in suspended cell cultures of chick embryo brain, chick embryo muscle, rabbit fibroma and rabbit myxoma as well as certain human tumor tissues. A loss of titer for mice was observed after a number of tissue culture passages. Normal adult rabbit tissues did not support the growth of Semliki Forest fever virus, but in some instances tissue of young (16, 30, 90-day-old) animals yielded positive results. Although there was no evidence of any interference with the fibroma virus on the part of Semliki Forest fever virus in tissue culture preparations, Semliki Forest fever virus did inhibit tumor cell proliferation in hanging drop cultures.

560. Elmendorf, T. E., and Smith, H. H.: Proc. Soc. Exper. Biol. & Med. **36**:171, 1937.
561. Jadin, J.: Ann. Soc. belge méd. trop. **17**:27, 1937.
562. Penna, H. A., and Moussatche, H.: Brasil-med. **53**:903, 1939.
563. Theiler, M., and Smith, H. H.: J. Exper. Med. **65**:787, 1937.
564. Smith, H. H.; Penna, H. A., and Paoliello, A.: Am. J. Trop. Med. **18**:437, 1938.
565. Fox, J. P., and Laemmert, H. W.: Am. J. Hyg. **46**:21, 1947.
566. Roca-Garcia, M.: J. Infect. Dis. **75**:160, 1944.
567. Ginder, D. R., and Friedewald, W. F.: Proc. Soc. Exper. Biol. & Med. **77**:272, 1951; ibid. **79**:615, 1952.

Embryonated Eggs.—"Anopheles A" virus was carried through 10 passages in 7-day chick embryos inoculated intraembryonically (Roca-Garcia [566]). Propagation of the Semliki Forest fever virus following this route of inoculation was also reported (Smithburn [568]). Taylor,[184] in a comprehensive study of the behavior of a number of African and South American viruses in the embryonated egg, demonstrated the propagation of "Anopheles A," Bunyamwera, Bwamba, Haemagogus A and B, Leucocelaenus, Ntaya, Sabethes, Semliki Forest fever, Uganda S and Zika viruses after either yolk sac or amniotic sac inoculation. In general, the chorioallantoic membrane and the allantoic cavity were found to be less satisfactory sites of inoculation. Of this group of viruses, only Ntaya and Semliki Forest fever viruses were consistently lethal for the embryos. The "Anopheles A and B," Bunyamwera, Bwamba, Ntaya, and Uganda S viruses were found in higher concentrations in the brains than in the bodies of the infected embryos after yolk sac inoculation, while the remainder of the viruses appeared to be pantropic.

The "Anopheles B" virus could be passed by yolk sac inoculation only after preliminary intracerebral egg passage, and positive results with the "Wyeomyia" virus were obtained only with brain-to-brain passage.

On the basis of neutralization (Smithburn [569]) and complement fixation (Kerr [570]) tests, it has been suggested that Haemagous A and B, Leucocelaenus, and Sabethes viruses are strains of Theiler's mouse encephalomyelitis virus.

568. Smithburn, K. C.: J. Immunol. **52**:309, 1946.

569. Smithburn, K. C.: J. Immunol. **68**:441, 1952.

570. Kerr, J. A.: J. Immunol. **68**:461, 1952.

News and Comment

American Society for Experimental Pathology.—The American Society for Experimental Pathology will hold its 1954 meeting in Atlantic City, N. J. Incoming officers are D. Murray Angevine, President; Russell L. Holman, Vice-President; Cyrus C. Erickson, Secretary-Treasurer; Harold L. Stewart and F. W. Hartman, Councilors.

American Association of Blood Banks.—The Sixth Annual Meeting of the American Association of Blood Banks will be held Oct. 17-20, 1953, at the LaSalle Hotel, Chicago. Detailed information may be obtained from the Office of the Secretary, 3500 Gaston Ave., Dallas 4, Texas.

New Bulletin on Tissue Transplantation.—The establishment of *Transplantation Bulletin* has been announced. Workers in the fields of medicine and biology who are interested in tissue transplantation are invited to submit their names and fields of interest to E. J. Eichwald, M.D., University of Utah College of Medicine, Salt Lake City, Utah.

Books

Nutrition and Diet in Health and Disease. By James S. McLester, M.D., Professor of Medicine Emeritus, University of Alabama, and William J. Darby, M.D., Ph.D., Professor of Biochemistry and Director of the Division of Nutrition, Vanderbilt University. Sixth edition. Price, $10.00. Pp. 710, with 14 illustrations and 145 tables. W. B. Saunders Company, 218 W. Washington Sq., Philadelphia 5, 1952.

McLester's "Nutrition and Diet in Health and Disease" is as much an institution among American physicians as Fannie Farmer's cook book is for their wives. However, the science of nutrition has come a long way since 1927 when Dr. McLester first brought out his book, and, to round out his broad clinical knowledge, artistry, and skill in the dietary management of the sick, he has chosen a collaborator. This is Dr. W. J. Darby, Professor of Biochemistry and Director of the Division of Nutrition at Vanderbilt University. Dr. Darby is well known all over the world for his many biochemical and clinical studies of nutrition in man and beast, and he has written most of the chapters on "Nutrition in Health," while the senior author has revised his chapters on "Nutrition in Disease." However, this division of work has not produced a dichotomy of aims, for, on reading through the book, it is quite clear that there has been a successful marriage of clinical and theoretic considerations of nutrition.

Since World War I, in this country we have seen extraordinary advances in food technology; in agriculture; in animal husbandry, and in transportation, storage, and distribution of food. Our chemists have discovered, synthesized and now produced in bulk at least a dozen vitamins. The economic standards of people have risen; bread and flour is fortified with necessary nutrients, and, through the efforts of the United States Public Health Service and the Food and Nutrition Board, educational programs have taught, and continue to teach, most of our population the importance of proper nutrition. These advances have for all practical purposes wiped out primary nutritional diseases, such as beriberi and pellagra. Our wealth, in fact, has led to wastage of food and to a sharp increase in obesity among our population. In the rest of the world, nutrition has been, and is still, disturbed as a result of war, leached soils, the depredations of insects, primitive agriculture, poverty, and ignorance. Hunger and starvation are the common lot of man in Africa, Asia, and much of Europe and South America. Thus it was surprising to find a rather skimpy treatment of the two common results of malnutrition in the chapter on "Obesity and Leanness." Other notable omissions relate to electrolyte disturbances, which in the past few years have become major problems in the dietary management of patients. The section "Sodium and Potassium in Health" is very short, and in the second part of the book the dangers of sodium restriction are dismissed in one sentence. Moreover, there is no mention of the recognition and management of the "low-salt" syndrome.

Despite these faults, the sixth edition of the book, with its detailed appendix, remains the best volume of its kind and should be kept close at hand by all who treat the sick.

A Manual of Clinical Allergy. By John M. Sheldon, M.D., Professor of Internal Medicine, University of Michigan Medical School; Robert G. Lovelle, M.D., Instructor in Internal Medicine, University of Michigan Medical School, and Kenneth P. Mathews, M.D., Assistant Professor of Internal Medicine, University of Michigan Medical School. Price, $8.50. Pp. 413, with 27 illustrations. W. B. Saunders Company, 518 W. Washington Sq., Philadelphia 5, 1953.

Allergy is in transition, and textbooks on allergy are rapidly outdated by the advances in fundamental research. Recent textbooks have emphasized the practical aspects of the field, rather than its theoretical problems. Within this group, this manual is a competent contribution to instruct physicians in, as the authors state in their preface, "doing a sound allergic workup and treating patients in a safe and medically accepted fashion." Even if one does not agree with every suggestion, one must admit that the book fulfills its purpose faithfully and with an unusual attention to detail. It goes so far as to include valuable advice on the planning and construction of an office for the management of allergic patients. The section on pollen identification

is outstanding because of its beautiful illustrations, prepared under the supervision of Mr. Oren C. Durham, with the assistance of Mr. Tom Jones and the staff of the Illustration Studios of the University of Illinois.

In emphasizing the value of the book, one must keep in mind that it teaches techniques used in allergy, not allergy itself. The manual will be most useful, therefore, to physicians who are already familiar with the principles upon which the practice of allergy is based. The stunning shadowgraph of a ragwed pollen on the cover of the book—produced with the most modern means of magnification—illustrates, in fact, the limitations of techniques; while intriguing, it shows indeed not more, and perhaps less, than can be seen with any ordinary microscope.

Building America's Health: America's Health Status, Needs, and Resources. Volume 2. A Report to the President by the President's Commission on the Health Needs of the Nation. Price, $1.25. Pp. 320, with 60 illustrations and 62 tables. United States Government Printing Office, Washington 25, D. C., 1952.

Building America's Health: America's Health Status, Needs, and Resources. Volume 3. A Statistical Appendix. A Report to the President by the President's Commission on the Health Needs of the Nation. Price, $1.50. Pp. 299, with 417 tables. United States Government Printing Office, Washington 25, D. C., 1952.

Building America's Health: Financing a Health Program for America. Volume 4. A Report to the President by the President's Commission on the Health Needs of the Nation. Price, $1.50. Pp. 363, with numerous illustrations and tables. United States Government Printing Office, Washington 25, D. C., 1952.

Klinische Fehldiagnosen. By Prof. Dr. M. Bürger, Direktor der Medizin, Universitätsklinik, Leipzig. Price, 58.50 German marks. Pp. 480, with 209 illustrations. Georg Thieme, Diemershaldenstrasse 47, (14a) Stuttgart-O; agents for U. S. A.: Grune & Stratton, Inc., 381 Fourth Ave., New York 16, 1953.

Lehrbuch der Krankheiten des Herzens und der Blustrombahn. By Prof. Dr. Fritz Lange. Price, 71 German marks. Pp. 631, with 192 illustrations. Ferdinand Enke, Hasenbergsteige 3, Stuttgart W., 1953.

Das Ulkus des Magens und Zwölffingerdarms als Problem eines Schutzstoffmangels. By Dr. H. Ramb, Chefarzt der Chirurg., Abteilung am Laurentius-Hospital Essen-Steele. Price, 6.50 German marks. Pp. 68, with 28 illustrations. Georg Thieme, Diemershaldenstrasse 47, (14a) Stuttgart-O; agents for U. S. A.: Grune & Stratton, Inc., 381 Fourth Ave., New York 16, 1953.

Osteosklerose und Knochenmarkfibrose. By Rudolf Stodtmeister, Dr. med. Dr. phil., apl. Professor für innere Medizin an der Universität Heidelberg, Chefarzt der Inneren Abteilung des Städtischen Krankenhauses Pforzheim; Stefan Sandkühler, Dr. med., Assistant der Ludolf-Krehl-Klinik (Medizin. Univ.-Klinik), Heidelberg; unter röntgenologischer Mitarbeit von Albert Laur, Dr. med., Assistant der Röntgenabteilung der Ludolf-Krehl-Klinik (Medizin. Univ.-Klinik), Heidelberg. Price, 28.50 German marks. Pp. 131, with 29 illustrations. Georg Thieme, Diemershaldrenstrasse 47, (14a) Stuttgart-O; agents for U. S. A.: Grune & Stratton, Inc., 381 Fourth Ave., New York 16, 1953.

Gynecological and Obstetrical Pathology. By Peter A. Herbut, M.D., Professor of Pathology, Jefferson Medical College, and Director of Clinical Laboratories, Jefferson Medical College Hospital, Philadelphia. Price, $12.50. Pp. 683, with 428 illustrations on 246 figures and 2 colored plates. Lea & Febiger, 600 S. Washington Sq., Philadelphia 6, 1953.

This textbook covers the assigned field in complete and detailed fashion. There are twelve chapters. The first covers the embryology of the female genital system. The second and third deal with the physiology and cytology of the genital tract and were written by A. E. Rakoff.

The next seven chapters deal in conventional fashion with the vulva, vagina, cervix, corpus uteri, fallopian tubes, ovaries, and fetal membranes, respectively. The last two chapters were written by L. A. Erf and discuss the Rh factor and blood groups, the maternal anemias, toxemia of pregnancy, and puerperal death. The chapters written by P. A. Herbut are each introduced with a review of the normal anatomy of the part, followed by the pathologic changes, which in turn are classified into congenital, inflammatory, neoplastic, and mechanical. Each disorder is then dealt with under the headings of definition, incidence, cause, gross and microscopic appearances, complications, clinicopathologic correlation, diagnosis, treatment, and prognosis. There is an adequate bibliography at the end of each chapter, but the references are almost solidly Anglo-Saxon and largely from the recent American literature. The text is well illustrated. The type is clear, with few typographical errors. The style is simple and terse.

This textbook is a suitable one for reference on the pathologist's and gynecologist's shelf. It is, however, too detailed and too long for the undergraduate student who can devote so little time to this subject and who prefers to have obstetrical and gynecologic pathology incorporated within a predominantly clinical text. For physicians who are specializing in this field and in pathology, this volume should be of enormous help in preparation for their specialty board examinations.

Pharmacology and Toxicology of Uranium Compounds: Chronic Inhalation and Other Studies. Edited by Carl Voegtlin, Ph.D., Lecturer in Cancer Research and Toxicology, University of Rochester School of Medicine and Dentistry; formerly, Chief of the Division of Pharmacology, National Institutes of Health, United States Public Health Service; formerly, Director of Cancer Research and Chief of the National Cancer Institute, and Harold C. Hodge, Ph.D., Professor of Pharmacology and Toxicology, University of Rochester School of Medicine and Dentistry. National Nuclear Energy Series, Division VI, Volume 1, Parts 3 and 4. Price, $18.00. Pp. 1,379, with numerous illustrations and tables. McGraw-Hill Book Company, Inc., 330 W. 42nd St., New York 18, 1953.

Parts 3 and 4 deal with the chronic effects of uranium and the mechanism of uranium poisoning, continuing and extending the material presented in Parts 1 and 2. Experiments extend over a period of one or two years. Methods and procedures are clearly and comprehensively given. The two volumes comprise chapters 18 to 28 inclusive, and an excellent résumé of these chapters is given by Hodge, one of the editors. Apparently the individual authors have had a free hand in presenting data. Bibliographies are extensive and are appended to each chapter. The make-up of the volumes is most pleasing; the type is clear (lithoprint), and charts and illustrations are numerous. The report is somewhat encyclopedic and certainly comprises the most authoritative writing on the pharmacology and toxicology of uranium. It can be recommended without question to those interested in this field.

An Atlas of Surgical Exposures of the Extremities. By Sam W. Banks, M.D., Associate Professor of Orthopedic Surgery, Northwestern University Medical School; Attending Orthopedic Surgeon, Chicago Memorial Hospital and Woodlawn Hospital, and Harold Laufman, M.D., Ph.D., Associate Professor of Surgery and Director of Experimental Surgery, Northwestern University Medical School; Associate Attending Surgeon, Michael Reese Hospital. Price, $15.00. Pp. 391, with 552 illustrations on 179 plates. W. B. Saunders Company, 518 W. Washington Sq., Philadelphia 5, 1953.

This atlas is an excellent presentation of the surgical approach to various areas of the extremities. Illustrations of surgical exposure of all of the joints and long bones of the extremities are given. The black and white illustrations are particularly good. They can be easily and quickly understood. A minimum number of legends on the drawings have been used most effectively. Each plate is accompanied by a description of the procedure illustrated. The descriptions point out indications for the use of the exposure and call attention to structures to be avoided or protected. In addition to the usual table of contents, each section has a separate table, which aids in the use of the book. The indexing is adequate. This book can be recommended as the authors intended to the student, the resident, and the experienced surgeon.

Clinical Diagnosis by Laboratory Methods: A Working Manual of Clinical Pathology. By James Campbell Todd, M.D., Late Professor of Clinical Pathology, University of Colorado School of Medicine; Arthur Hawley Sanford, M.D., Emeritus Professor of Clinical Pathology, The Mayo Foundation, University of Minnesota; Emeritus Member, Division of Clinical Laboratories, The Mayo Clinic; Director of Laboratories, Rochester State Hospital, and Benjamin B. Wells, M.D., Ph.D., Professor of Medicine, Department of Medicine, University of Arkansas School of Medicine. Twelfth edition. Price, $8.50. Pp. 998, with 946 illustrations, 197 in color, on 403 figures. W. B. Saunders Company, 218 W. Washington Sq., Philadelphia 5, 1953.

Five years have elapsed since the last edition of this classic medical text. For the new edition Dr. Benjamin Wells has been given the place of co-author. The present volume follows the general format of its immediate predecessor, with no major alterations, but with numerous changes to bring the techniques and methods up to date. The text is increased by about forty pages, with extensive additions in chapters on the blood and on serology. There have been numerous changes in the matter of illustrations, many of the older ones having been omitted and some new ones added. The reviewer feels that many of the pictures of common apparatus and instruments could advantageously be omitted. The text is clear and lucid, and the volume is very highly recommended.

The Roentgen Aspects of the Papilla and Ampulla of Vater. By Maxwell H. Poppel, M.D., F.A.C.R., Professor of Radiology, Post-Graduate Medical School of the New York University-Bellevue Medical Center; Director of the Roentgen Ray Department, Bellevue Hospital; Consultant in Radiology, Veterans Administration Hospital, Bronx, N. Y.; Consulting Radiologist, United States Naval Hospital, St. Albans, Long Island; Roentgenologist, New York University Hospital; Harold G. Jacobson, M.D., F.A.C.R., Director, Department of Roentgenology, Hospital for Special Surgery; Associate Clinical Professor of Radiology, Post-Graduate Medical School of the New York University-Bellevue Medical Center, New York; Attendant in Radiology, Veterans Administration Hospital; formerly, Chief, Radiology Service, Veterans Administration Hospital, Bronx, N. Y., and Robert W. Smith, M.D., Senior Resident in Radiology, Veterans Administration Hospital, Bronx, N. Y. Price, $8.50. Pp. 195, with 106 figures and 8 tables. Charles C Thomas, Publisher, 301-327 E. Lawrence Ave., Springfield, Ill., 1953.

The Physical Examination of the Surgical Patient. By J. Englebert Dunphy, M.D., F.A.C.S., Associate Clinical Professor of Surgery, Harvard Medical School, and Thomas W. Botsford, M.D., F.A.C.S., Clinical Associate in Surgery, Harvard Medical School. Price, $7.50. Pp. 326, with 188 figures. W. B. Saunders Company, 218 W. Washington Sq., Philadelphia 5, 1953.

Virus and Rickettsial Classification and Nomenclature. Edited by Roy Waldo Miner, with contributions by 27 authors. Price, $4.00. Pp. 242, with illustrations, charts, and tables. Annals of the New York Academy of Sciences, Volume 56, Article 3. The New York Academy of Sciences, 2 E. 63rd St., New York 21, 1953.

The Harvey Lectures: Delivered Under the Auspices of The Harvey Society of New York, 1951-1952. Series 47. By A. R. Todd, F. R. Winton, H. W. Magoun, W. Barry Wood, Jr., I. L. Chaikoff, Carroll M. Williams, Louis B. Flexner, Walter H. Seegers, and L. Zechmeister. Price, $7.50. Pp. 271, with numerous figures, graphs, and charts. Academic Press, Inc., 125 E. 23rd St., New York 10, 1953.

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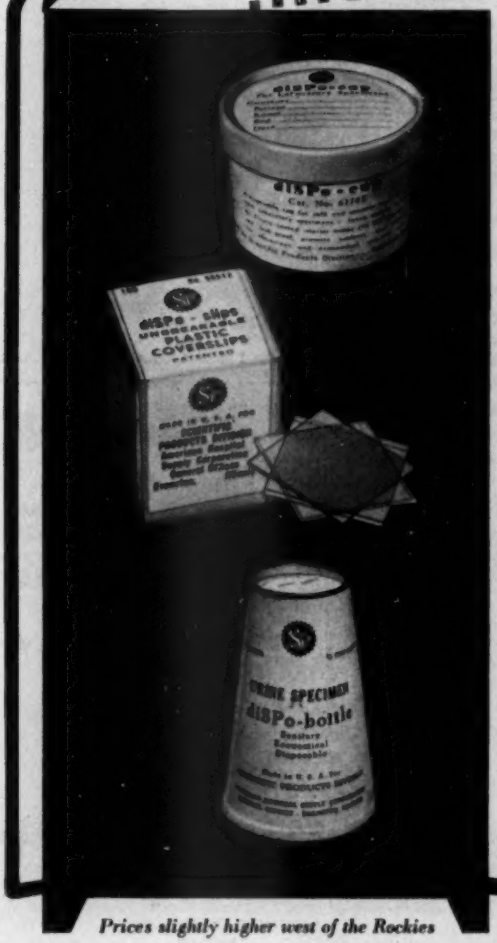

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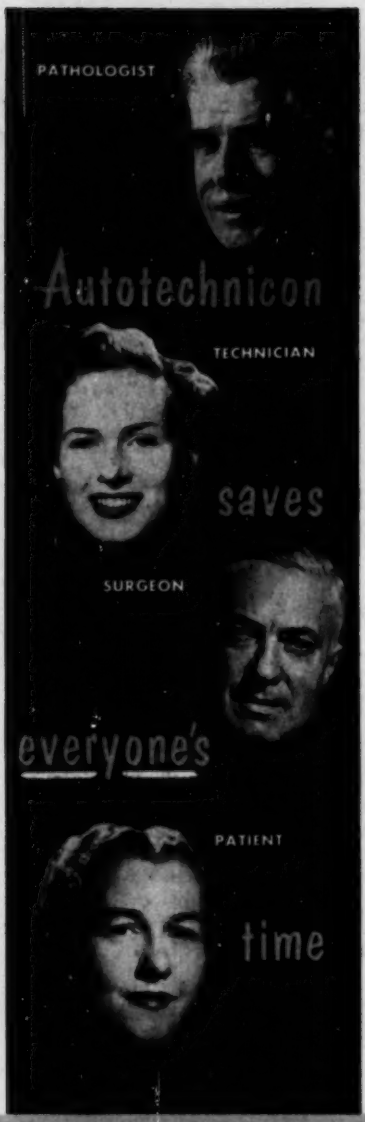

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